

MYOCARDIAL INTERMEDIARY METABOLISM

with special reference to  
the isolated, contracting, perfused rat heart.

A thesis submitted to the University of Cape Town  
for

the Degree of Doctor of Medicine

by

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The heart has its reasons,  
which reason does not know.

Pascal, *Pensées*, Pt. II, Art xvii, No. 5



## CONTENTS

		page
	List of figures	
	List of tables	
	Abbreviations	
	Acknowledgments	
	ABSTRACT	i
Chapter 1	HISTORICAL BACKGROUND AND REVIEW OF THE LITERATURE	1
Chapter 2	METHODS	4
	Apparatus	4
	Mounting of hearts	5
	Perfusate	7
	Carbohydrate analyses	9
	Lipid analyses	11
	Isotopic analyses	13
	Expression of results	14
	Preparation of insulin	14
	Scope of this thesis	15
Chapter 3	CHARACTERIZATION OF EXPERIMENTAL SYSTEM	16
	Adequacy of oxygenation	16
	Type and Length of pre-perfusion	18
	Nutritional state of donor rats	19
	Recovery of label	20
	Conclusions	21
Chapter 4	HEXOSE METABOLISM	22
	Uptake and fate of glucose	22
	Effect of insulin	25
	Mannose and fructose	26
	Summary	28



<b>Chapter 5</b>	<b>FREE FATTY ACID METABOLISM</b>	<b>29</b>
	Cardiac performance	29
	Effect of perfusion technique on F.F.A.	30
	Method of adding label	30
	Comparative metabolism of palmitate 1-, 6-, and 11- $C^{14}$	33
	Effect of perfusate palmitate concentration	33
	Oleic acid metabolism	34
	Conclusions	34
 <b>Chapter 6</b>	 <b>INTERACTION OF GLUCOSE AND PALMITATE METABOLISM</b>	 <b>36</b>
	Addition of palmitate to glucose-u- $C^{14}$	36
	Palmitate effect in fed state	37
	Effect of palmitate on glucose uptake	37
	Effect of palmitate on lipid formation from glucose-u- $C^{14}$	38
	Effect of glucose on palmitate- 1- $C^{14}$	39
	Glucose-F.F.A. interrelations in other systems	39
	Summary	43
 <b>Chapter 7</b>	 <b>MECHANISM OF GLUCOSE-PALMITATE INTERACTION</b>	 <b>44</b>
	Effect of palmitate, pyruvate and acetate on glucose-u- $C^{14}$	44
	Effect of glucose and acetate on palmitate-1- $C^{14}$	45
	Effect of palmitate on pyruvate- 1- $C^{14}$	46
	Summary	49
 <b>Chapter 8</b>	 <b>EFFECT OF ANOXIA, HYPOTHERMIA, EPI- AND NOR-EPINEPHRINE ON MYOCARDIAL INTERMEDIARY METABOLISM</b>	 <b>50</b>
	A. Effect on Carbohydrate Metabolism	50
	Anoxia	50
	Norepinephrine	52
	Perfusion at 28°C.	53



B. Effect on Palmitate Metabolism	54
Anoxia	54
Perfusion at 28°C.	54

C. Summary	54
------------	----

Chapter 9	REVIEW OF DATA	56
-----------	----------------	----

## References



# KEY TO FIGURES

<u>Figure</u>	<u>Title</u>	<u>Followed by page</u>
1	Left: washout system Right: closed re-circulation system	4
2-6	Mounting of hearts	5
7	Time course of rat heart perfusion	7
8	Schema of analytical procedures	9
9	Enzymatic lactate method	11
10	Parameters measured	15
11	Schema of cardiac energetics	15
12	Effect of method of oxygenation (1) Performance	17
13	Effect of method of oxygenation (2) Metabolic behaviour	17
14	Nutritional state: (1) Blood sugar, plasma F.F.A. and cardiac glycogen	19
15	Nutritional state: (2) Glucose metabolism	19
16	Nutritional state: (3) Palmitate metabolism	20
17	Glucose metabolism: (1) Uptake	22
18	Glucose metabolism: (2) Uptake, fed and fasted	22
19	Glucose metabolism: (3) CO <sub>2</sub> production	23
20	Glucose metabolism: (4) Glycogen formation	23
21	Glucose metabolism: (5) Lactate production	24
22	Hexose metabolism: Mannose and fructose	26
23	Palmitate metabolism: (1) Addition of label	30
24	Palmitate metabolism: (2) Various positions of label	32
25	Palmitate metabolism: (3) Effect of concentration	33
26	Oleic acid metabolism	34
27	Glucose metabolism: (6) Effect of palmitate	36
28	Possible effect of fasting	37
29	Glucose metabolism: (7) Palmitate and insulin; effect on uptake	37
30	Glucose metabolism: (8) Lipid formation	38
31	Palmitate metabolism: (4) Effect of glucose	39
32	Effect of feeding and fasting on myocardial metabolism <u>in vivo</u>	40
33	Glucose metabolism: (9) Effect of palmitate, pyruvate and acetate	44
34	Palmitate metabolism: (5) Effect of glucose and acetate	45
35	Pyruvate metabolism: (1) Effect of palmitate	43
36	Pyruvate metabolism: (2) Effect of palmitate	47
37	Glucose metabolism: Effect of epi- and nor- epinephrine, oxythianine- treatment, and 28°C.	50
38	Palmitate metabolism: (6) Effect of anoxia and 28°C.	54



# KEY TO TABLES

<u>Table</u>	<u>Title</u>	<u>Followed by Page</u>
1	Composition of buffer	7
2	pH of perfusate	7
3	Analytical techniques	10
4	Lactate removal in F.F.A. titration	11
5	Dry/wet weight ratios	14
6	Heart nitrogen.	14
7	Effect of pre-perfusion	18
8	Recovery of carbohydrate: glucose 5 mM/L.	21
9	Recovery of carbohydrate: various glucose concentrations	21
10	Insulin effect on glucose metabolism	25
11	Effect of glucose on insulin-sensitivity of fructose-u-C <sup>14</sup>	27
12	F.F.A. binding by albumin	31
13	Effect of palmitate (300 uM/L.) on glucose-u-C <sup>14</sup> (7.5 uM/L.) in fed state	38



### ABBREVIATIONS

The following abbreviations have been used:

Acetyl CoA - Acetyl coenzyme A  
Acy CoA - fatty acid esterified to coenzyme A  
A.D.P. - adenosinediphosphate  
A.T.P. - adenosinetriphosphate  
CO<sub>2</sub> - carbon dioxide  
D.P.N. and D.P.N.H. - oxidized and reduced forms of diphosphopyridine nucleotide  
E.D.T.A., sodium - disodium salt of ethylene diamine tetra-acetic acid dihydrate  
F.F.A. - free fatty acids (= U.F.A. or unestrified fatty acids = N.E.F.A. or nonesterified fatty acids)  
Glucose-u-C<sup>14</sup> - randomly-labelled glucose  
N<sub>2</sub> - nitrogen  
O<sub>2</sub> - oxygen  
T.P.N. and T.P.N.H. - oxidized and reduced forms of triphosphoryridine nucleotide.  
uM. C6/g.w.w./30 min. - micromoles glucose equivalent per gram wet weight per 30 minute perfusion

Abbreviations accepted by Webster's New International Dictionary are given as such and not underscored. Examples are: ad lib., cf., et al.

In giving references in the text, the practice of The Lancet was followed.

In the figures the following phrases were used:

Glucose to CO<sub>2</sub> = Incorporation of glucose carbon into CO<sub>2</sub>, measured by incorporation of label from glucose-u-C<sup>14</sup> into labelled CO<sub>2</sub>;

Glucose to Glycogen = Incorporation of glucose carbon into glycogen, measured by incorporation of label from glucose-u-C<sup>14</sup> into labelled glycogen.



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ABSTRACT

Studies of myocardial metabolism in vivo have been considerably advanced by the introduction of coronary sinus catheterization. By determining the coronary arterio-venous differences of various substrates, Bing and other workers (Goodale, Olson) have been able to describe the overall picture of myocardial metabolism in man and intact experimental animals. This technique does not, however, allow adequate pinpointing of the precise pathways of cardiac metabolism in health and disease, and Bing concludes a recent review (1958) by advocating further studies using newer techniques such as radio-isotopes in a controlled in vitro system.

The isolated perfused rat heart preparation, as developed by Fisher and his associates at Oxford, was selected as a useful preparation for the study of myocardial intermediary metabolism. In order to measure radio-active carbon dioxide ( $C^{14}O_2$ ) formation from  $C^{14}$ -labelled substrates, Fisher's preparation was modified and a closed system designed. This consisted of 15 ml. Krebs-Henseleit bicarbonate medium re-circulated by a modified pumping system, with a gaseous phase of about 100 ml., consisting of 95% oxygen and 5% carbon dioxide. This preparation has the great advantage of allowing accurate assessment of the fate of any substrate taken up by the heart during the perfusion period. There are also other features which make isolated perfused rat heart a particularly attractive preparation. First, it can be maintained visibly functioning (i.e. contracting) for adequate periods of time. Moreover, the nature of the perfusing fluid can be varied at will, thus altering the substances reaching the myocardial cells along the natural vascular channels. Various hormones and drugs can



be added at will to the perfusing fluid and the effect on radio-active substrates studied. The small size of the rat heart is a real advantage. The size of the perfusion apparatus is small, which allows the simultaneous operation of four perfusion systems. Up to twenty-eight perfusions can be carried out in one day to study four or more variables in a controlled fashion.

As a test of the validity of this modified system, the coronary flow, force of heart beat and cardiac rate obtained with the closed system were compared with the values found with the more conventional system employing continuous oxygenation. Adequacy of oxygenation was assessed by these parameters and by the metabolic behaviour of the heart, and found to be adequate. It was demonstrated that a functionally stable preparation was achieved for the duration of the perfusion period in the closed system.

The standard perfusion time was thirty minutes, after an initial period of pre-perfusion with plain Krebs-Henseleit bicarbonate buffer. This pre-perfusion washed out blood from the coronary circulation, and allowed stabilization of cardiac performance. Experiments with varying lengths of pre-perfusion (from five to fifteen minutes) and with both plain buffer and buffer containing glucose 5 mM/L., showed that pre-perfusion with plain buffer for five minutes was the method of choice, since it resulted in the highest glucose uptake and  $C^{14}O_2$  formation in the subsequent perfusion period. Because the pre-perfusion was substrate-free, there was a decided glycogen-breakdown during this period, an advantage because it minimized the possible effects of endogenous glycogenolysis on the uptake and fate of exogenous carbohydrate during the actual perfusion period.



The majority of the techniques used for analysis of carbohydrate and lipid were those employed for the study of adipose tissue in the Baker Clinic Research Laboratory in the Department of Medicine, Harvard Medical School. These were modified for application to heart muscle, and a few additional techniques added; in particular, a rapid accurate enzymatic method of lactate determination was used.

#### A. CARBOHYDRATE METABOLISM

A major factor in the control of the uptake and fate of randomly-labelled glucose was the nutritional state of the donor rat. Hearts from non-fasted rats took up more glucose than hearts from rats fasted overnight or for four days; both the proportion and the absolute amounts of  $\text{CO}_2$  and glycogen formation from glucose, were greater in the fed state.

Glucose uptake occurred even at the lowest measured glucose concentrations (0.5 mM/L); no glucose threshold could be demonstrated. At such low concentrations there was a minimum of glycogen and lactate formation and a greater percentage of the glucose label could be recovered as  $\text{C}^{14}\text{O}_2$  than at high concentrations.

Myocardial handling of randomly-labelled mannose and fructose was also studied. Mannose metabolism resembled that of glucose. Fructose uptake was only one-fifth that of glucose, making it unlikely that fructose itself is a fuel for cardiac contraction.

The effect of insulin on the metabolism of glucose, mannose and fructose was also studied. In every case the hexose uptake was increased by about 50%. The fate of the increased uptake was variable, but glycogen synthesis was more constant than increased  $\text{CO}_2$  or lactate formation.



## B. FREE FATTY ACID METABOLISM

The free fatty acid (F.F.A.) metabolism of the rat heart was studied in detail by the use of albumin-bound long-chain fatty acids.

Studies with palmitate variously labelled with  $C^{14}$  in positions 1, 6, and 11, showed that the metabolism of palmitate was such that the fate of the C-1 label was a reasonably accurate index of the handling of all sixteen carbon atoms of the chain.

The problems raised by the albumin-palmitate binding are analyzed. The difficulties of interpreting data obtained with the labelled palmitate were demonstrated by different myocardial uptake of labelled and titratable palmitate.

The titratable palmitate uptake was linearly related to perfusate concentrations up to 1,200  $\mu M/L.$ , a value above the plasma F.F.A. level of rats fasted for four days. Using albumin-bound palmitate labelled in a specified fashion, the major part of the label (60-75%) was incorporated into  $C^{14}O_2$ , and most of the rest into the tissue fatty acid fraction. The effect of feeding and fasting donor rats on palmitate metabolism was minimal, in contrast to the marked influence of nutrition on glucose metabolism.

Oleic acid metabolism was studied as an example of an unsaturated long-chain fatty acid. There was a slightly greater uptake of oleate than palmitate, but the overall fates of the label in oleate-1- $C^{14}$  and in palmitate-1- $C^{14}$  were similar.

## C. INTERRELATION OF CARBOHYDRATE AND LIPID METABOLISM

Glucose metabolism of the isolated heart is so profoundly influenced by the simultaneous presence of palmitate in the perfusate, that long-chain free fatty acid qualifies as a major factor in the control of the handling



of glucose by the heart. A uniformly constant effect of the palmitate was the marked inhibition of glucose oxidation in all conditions of this study. Under specified conditions the palmitate exerted three other effects; (i) lactate formation was increased; (ii) the incorporation of  $C^{14}$ -label from glucose into glycogen was increased and there was more glycogen in the heart at the end of the perfusion; and (iii) there was inhibition of the insulin-induced increase of glucose uptake. The inhibition of the glucose oxidation by palmitate was least in conditions closely simulating those found in rats fed ad lib.

In marked contrast, the addition of glucose (5 and 10 mM/L.) to palmitate-1- $C^{14}$  (400 and 800  $\mu$ M/L.) had little influence on palmitate oxidation, especially in the fasted state. The added glucose did not affect palmitate uptake, but it did increase the amount of the label recovered in tissue fatty acid; evidence is presented to show that tissue glyceride-glycerol formation was probably encouraged.

In order to elucidate the fatty acid-glucose interaction, the following were studied: (a) the effect of acetate (5 mM/L.) on glucose-u- $C^{14}$  and palmitate-1- $C^{14}$  oxidation; (b) the effect of pyruvate (5 mM/L.) on glucose oxidation; (c) the effect of palmitate on pyruvate-1- $C^{14}$  oxidation. The effect of palmitate in reducing incorporation of label from carboxyl-labelled pyruvate (5 mM/L.) was most marked in the fasted state. This finding, although it offered difficulties of interpretation, provides a plausible site for the action of palmitate on glucose metabolism.

These studies support the thesis that long-chain free fatty acid inhibition of glucose oxidation is a real phenomenon and not due to isotope dilution. The preferential utilization of free fatty acids, when available



to the heart, may provide an explanation for the puzzling increase of cardiac glycogen in fasting and diabetes, in contrast to the fall of glycogen in liver and diaphragm under these conditions.

#### **D. EFFECTS OF ANOXIA, HYPOTHERMIA AND NOREPINEPHRINE**

Anoxia promoted glucose uptake and glycogen breakdown with liberal lactate production and little incorporation of label from glucose into glycogen or  $C^{14}O_2$ . On the other hand, palmitate uptake was decreased by anoxia but a greater proportion of label was found in tissue fatty acid.

Perfusion at 28° C. (instead of the normal 38° C.) decreased glucose synthesis. Hypothermia did not affect palmitate uptake, but reduced  $C^{14}O_2$  formation.

Norepinephrine ( $10^{-5}$  M/L.) markedly stimulated cardiac rate, force and coronary flow. The glucose uptake was increased, as were glycogen breakdown and lactate formation. The physical performance of the heart and the vigorous  $CO_2$  formation from glucose distinguished the effects of norepinephrine from those of anoxia.

#### **E. PATHWAYS OF INTERMEDIARY METABOLISM**

Some pathways of myocardial intermediary metabolism are delineated by the data of this study: (i) the oxidation of free fatty acids in preference to glucose; (ii) the control of glucose metabolism by free fatty acids, with suggestion of a block at the site of entry of pyruvate into the Krebs cycle; (iii) the slight effect of glucose on free fatty acid oxidation; (iv) the glucose-free fatty acid interaction at the triglyceride level, without any resulting influence on the oxidation of either glucose or free fatty acids; (v) the minimal transformation of



glucose into fatty acids within the heart (possibly related to a poorly developed hexose-monophosphate shunt), and the negligible conversion of free fatty acids into glycogen.

**CONCLUSION:**

These studies on the isolated perfused rat heart support those executed in other systems. They delineate the key role of lipids in cardiac metabolism, but also emphasize the importance of carbohydrate as cardiac fuel in the fed state. The nutritional state of the donor rat and the presence of long-chain free fatty acid are two of the major factors regulating glucose metabolism of the isolated heart: the fed state promotes glucose uptake and oxidation, while palmitate diverts glucose from oxidation to other fates.



CHAPTER ONE**HISTORICAL BACKGROUND AND REVIEW OF THE LITERATURE**

Some of the earliest studies of myocardial metabolism used the isolated perfused mammalian heart, which is essentially the preparation used for the work presented in this thesis. Langendorff, in 1895, described a perfusion technique which was subsequently used by Locke and Rosenheim (1907) to establish glucose uptake by heart muscle.

The description of the heart-lung preparation from the dog, by Knowlton and Starling (1912 a), stimulated interest in the metabolism of the dog's heart. These workers rapidly established (1912 b) that sugar was used by the dog heart and that pancreatectomy impaired this power of "consuming sugar". On attempting to repeat these findings, Patterson and Starling (1913) found marked variations in the glucose uptake; this they ascribed to breakdown of cardiac glycogen interfering with the glucose uptake. Lovatt Evans and his co-workers (1935) used an isolated heart-oxygenator system, and agreed that glucose uptake by the heart was significant. The lactate uptake of their preparation was even higher than that of glucose, but the blood lactate levels were correspondingly higher. Even if all the glucose and lactate taken up were oxidized, the cardiac oxygen usage could not be accounted for. This agreed with the earlier suggestion by Evans (1914) that only about one-third of the heart's energy is supplied by carbohydrate oxidation. Furthermore, Cruickshank and McClure (1936) suggested that in certain circumstances the "direct combustion" of fat might supply energy for the heart. In 1941 Cruickshank



and Kosterlitz used the rat heart-lung preparation to confirm fat usage by the heart, and stated that "the primary source of its energy, in so far as it is derived from fat, is the blood fatty acids."

These earlier workers therefore delineated two of the most important cardiac fuels: carbohydrate and fatty acids. But these findings could not be extended to man before the introduction of coronary sinus catheterization by Bing and his colleagues in 1947, and the use of this method to study coronary blood flow and myocardial metabolism in vivo (Goodale et al., 1948). The desirability of data on cardiac arterio-venous differences had been impressed on Goodale by his close contact with Drs. Burwell and Dexter during the study of a case of beri-beri heart disease at the Peter Bent Brigham Hospital (Burwell and Dexter, 1947). By determining the coronary arterio-venous differences of various substrates Bing, Olson and Goodale have been able to describe the overall myocardial metabolism in man and intact experimental animals. In addition to establishing that the chief energy sources of the intact human heart are carbohydrate and fatty acids (Bing et al., 1953; Bing et al., 1954), this type of work promoted much interest in this field. The increased cardiac uptake of fatty acids and ketones in fasting and diabetes was described and confirmed (Goodale and Hackel, 1953; Ungar et al., 1955; Goodale et al., 1959). Further studies (Blain et al., 1956) supported the earlier suggestion of Olson and Schwartz (1951) that heart failure could be divided on a biochemical basis into: (a) disturbances of energy production, as in beri-beri and thyrotoxicosis; and (b) disturbances of energy utilization, into which category they placed congestive heart failure.



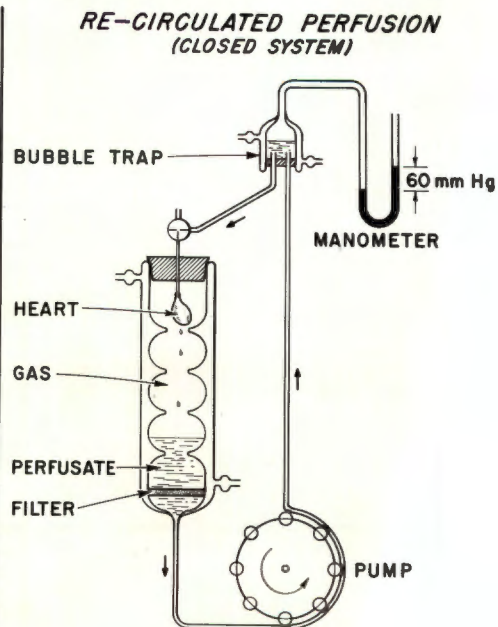
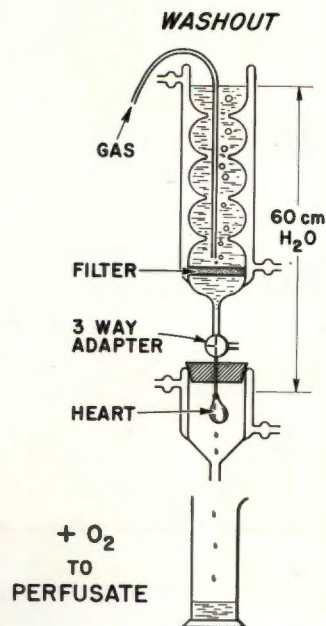
Studies of coronary arterio-venous differences do not, however, permit adequate pinpointing of the precise pathways of cardiac metabolism in health and disease, and Bing's group concludes their recent review (1958) by advocating further studies using newer techniques, such as radio-isotopes, in a controlled in vitro system.

Radio-active isotopes have been used to study the metabolism of pyruvate, lactate and acetate by ventricular slices (Pearson et al., 1949; Olson and Stare, 1951). Vigorous oxidation of all these substrates occurred, but the factors controlling their metabolism have not been defined, nor have radio-isotopes been fully exploited. Furthermore tissue slices need not accurately reflect the metabolism of the whole organ. There are also the difficulties of maintaining a viable preparation, since glycogen is rapidly lost from the centre of the slice and progressive deterioration of structure occurs (Pearson et al., 1949).

Another line of approach was developed by Fisher, working in the Department of Biochemistry at Oxford. Fisher and co-workers revived the Langendorff technique and introduced technical improvements which yielded a preparation stable for adequate time periods; their chief interest was to study the effect of insulin (Bleehen and Fisher, 1954). The measurement of small changes in the perfusing fluid was facilitated by the introduction of a technique permitting re-circulation of a small volume of perfusate (Morgan et al., 1961). This preparation is the one that has been adopted for use in this study.



# **RAT HEART PERFUSION**



**Fig. 1 (left)**  
Washout or  
pre-perfusion  
system

**Fig. 1 (right)**  
Closed re-circulation  
system



## CHAPTER TWO

### METHODS

The isolated, perfused rat heart is a particularly attractive preparation with which to study heart muscle metabolism. First, it can be maintained visibly functioning (i.e. contracting) for adequate periods of time. Secondly, the perfusing fluid reaches the intact myocardial cells along the natural channels. Thirdly, the nature of the perfusing fluid can be varied at will. Fourthly, the small size of the rat heart is a real advantage, allowing the simultaneous operation of four perfusion systems. Up to twenty-eight perfusions can be carried out in one day to study four or five variables in a controlled fashion.

#### (a) APPARATUS

The perfusion apparatus is basically very simple. The isolated heart first undergoes an initial washout (Fig. 1, left) to rid it of blood, and is then kept viable by a suitably oxygenated medium which is circulated (Fig. 1, right) through the aorta, coronary arteries and veins, and returns to the atria to fall out of the severed great veins. The emerging perfusate drops from the heart through a coarse porosity sintered glass filter, which is essential to maintain coronary flow (Bleehen and Fisher, 1954). In the system usually used, the medium is continuously gassed with 95% oxygen ( $O_2$ ) and 5% carbon dioxide ( $CO_2$ ). As this does not permit ready measurement of  $C^{14}O_2$  formation from radio-active substrates, Drs. B. le Boeuf and J. Shipp of the Baker Laboratory, suggested



## MOUNTING OF HEARTS

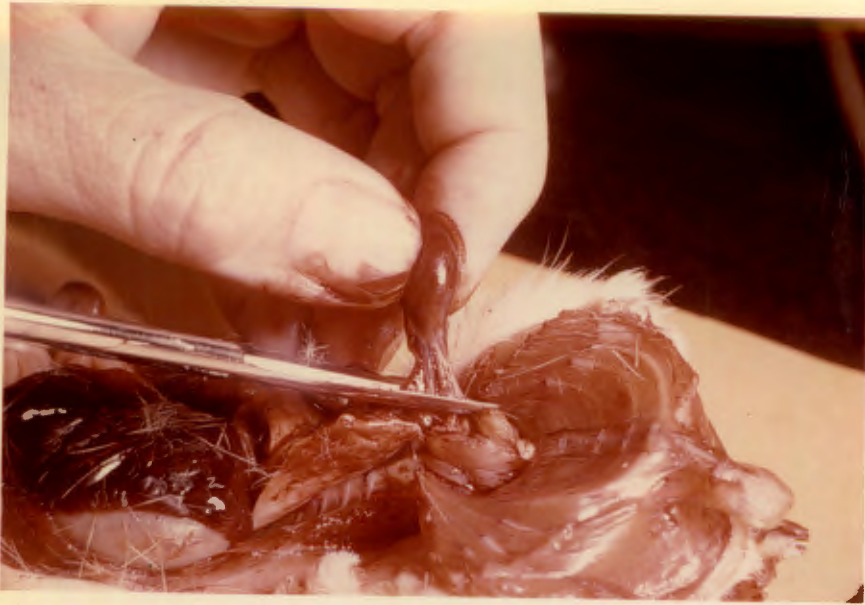


**Fig. 2** Albino rats were sacrificed by decapitation.



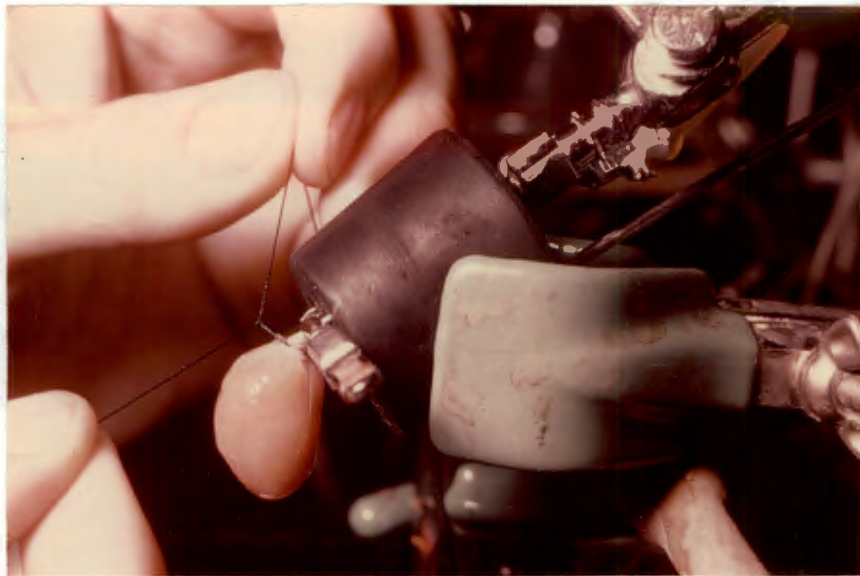
**Fig. 3**  
The thoracic  
cavity was  
widely exposed.





**Fig. 4**  
Heart lifted  
between fore-  
finger and  
thumb.

**Fig. 5**  
Aorta tied  
onto cannula.



**Fig. 6**  
Mounting  
completed.



that the system be closed after the initial gassing.

This closed system is the re-circulation system used in these studies. It consisted of 15 ml. of bicarbonate buffer re-circulated by a peristaltic pump\*, with a gaseous phase of 100 ml. of  $O_2 - CO_2$  (95:5%).

#### (b) MOUNTING OF HEARTS

Male albino rats (Harvard Biologic Laboratory or Charles River Breeding Company), weighing between 200 and 250 grams, were sacrificed by decapitation (Fig. 2). No anticoagulant was used. The thoracic cavity was widely opened (Fig. 3), after which the heart was gently lifted between forefinger and thumb (Fig. 4), and the great vessels severed. The heart was then placed in bicarbonate buffer at  $4^{\circ}C$ . After all contractions had ceased, the heart was mounted on a grooved number 18 gauge cannula filled with buffer by gravity from an elevated reservoir. The heart was held in position with a small arterial clamp placed across the upper part of the aorta while it was tied on with surgical thread (Fig. 5). The whole process from decapitation to mounting took less than two minutes.

Thereafter the heart was washed out with bicarbonate buffer gassed with  $O_2 - CO_2$  (95:5%) under pressure of about 45 mm. Hg. and at a temperature of  $37^{\circ}C$ . The heart, which had ceased beating while in the cold solution, began to contract and coronary flow resumed (Fig. 6), usually in less than thirty seconds. This initial washout is referred to as the preliminary or pre-perfusion, and it usually lasted five minutes.

\* Model No. 5-8950, American Instrument Company, Silver Springs, Maryland.



After the pre-perfusion in the washout unit, the heart was rapidly transferred to the closed re-circulation unit. The three-way adaptor was adjusted to begin the re-circulated perfusion with the buffer and substrate, which had received at least ten minutes thorough gassing with  $O_2 - CO_2$  (95:5%). This gas mixture had been equilibrated with water at  $37^\circ$ .

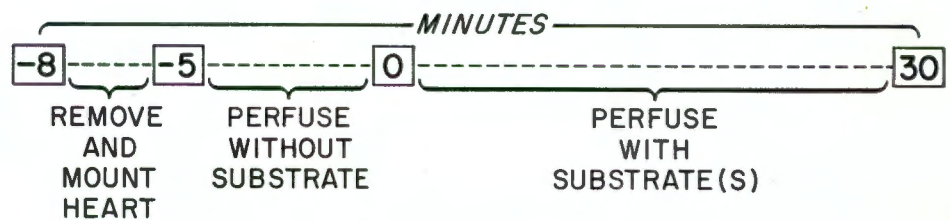
In the re-circulation unit the heart was suspended from the cannula in a water-jacketed chamber of  $2 \times 20$  cm. internal dimensions. The rubber stopper through which the cannula passed served both to suspend the heart and to close off the equilibrated re-circulation unit from the atmosphere. The effluent drops from the heart equilibrated with the gaseous phase as they flowed down the fluted inner surface of the heart chamber. They then mixed with the perfusate above the sintered glass disc which filtered the perfusate before it was re-circulated through narrow bore 'tygon' tubing (American Instrument Company). It was essential that the tubing be biologically inert, as some types of tubing which contain organic tin, depress cardiac function within 20 minutes (Meyler et al., 1960).

In order to prevent bubbles from entering the coronary arteries, a water-jacketed 10/30 mm. tapered ground glass female unit was placed in the perfusion system just before the heart chamber, and was connected to a suitable manometer. The perfusion pressure of 60 mm. Hg. was empirically chosen as one which produced high rates of coronary flow.

Between each perfusion, the interior of the re-circulation unit was thoroughly washed with copious amounts of distilled water, and then with 5 ml. of the actual perfusate before the 15 ml. of perfusate



### TIME COURSE OF RAT HEART PERFUSION



**Fig. 7**

"Perfuse without substrate" = initial washout or pre-perfusion period.

"Perfuse with substrate" = actual perfusion in closed recirculation system.



TABLE 1MODIFIED KREBS-HENSELEIT BUFFER (K-H BICARBONATE)

	<u>grams/L.</u>	<u>mEq./L.</u>
NaCl	6.9252	118.5
NaHCO <sub>3</sub>	2.0899	24.9
KCl	0.3533	4.74
KH <sub>2</sub> PO <sub>4</sub>	0.1614	1.18
CaCl <sub>2</sub> · 6H <sub>2</sub> O	0.2781	1.27
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.1461	0.59
Na <sub>2</sub> SO <sub>4</sub>	0.0841	0.59

TABLE 2pH BEFORE AND AFTER PERFUSION

<u>Heart No.</u>	<u>Substrate</u>	<u>Initial pH</u>	<u>Final pH</u>
71	glucose 5 mM.	7.43	7.46
72		7.46	7.30
862	pyruvate 5 mM.	7.51	7.48
863		7.39	7.39
866	pyruvate 5 mM. plus palmitate 750 μM/L.	7.42	7.38
867		7.46	7.42

(mM. = mM/L.)



was added. An estimate of the amount of fluid left after cleaning the unit was made by measuring the dilution of a concentrated glucose solution. From 0.5 to 0.7 ml. of fluid remained. This agrees well with the measurements of Morgan et al. (1961) using an isotope dilution technique. The actual volume of the circulating perfusate was therefore 0.5 to 0.7 ml. greater than the 15 ml. initially inserted. No correction was made for this constant error of less than 5%.

Once in the closed system, the gross performance of the heart was monitored by counting the heart rate and by visually gauging the force of contraction; furthermore the coronary flow was measured by the rate at which the perfusate dripped from the heart (1 ml. =  $9.4 \pm 0.3$  drops).

The usual time course of the rat heart perfusion is shown in Fig. 7.

### (c) PERFUSATE

The buffer used was modified from that used by Krebs and Henseleit (1932). The exact composition is shown in Table 1. The reductions in calcium and magnesium concentrations were based on the unpublished observations of Fisher that the force of cardiac contraction was best maintained at the stated concentrations.

The radio-active substrates used were chiefly glucose- $u-C^{14}$  (New England Nuclear, Boston, Mass.) and palmitic acid- $1-C^{14}$  (Volk Radio Chemical Company, Chicago, Ill.). In addition, the following were used: fructose- $u-C^{14}$  (New England Nuclear), mannose- $u-C^{14}$  (Volk), palmitic acid- $6-C^{14}$  and - $11-C^{14}$  (Radio-Isotope Specialities, Burbank, Calif.), and oleic acid- $1-C^{14}$  (Radio-Isotope Specialities). All these materials were chromatographically pure. Radio-active glucose,



mannose, fructose and pyruvate were diluted with 'cold' carrier soon after their arrival in the laboratory, and then kept frozen. Radio-active palmitic and oleic acids were converted to the sodium salt and then kept frozen until use.

Albumin-fatty acid mixtures were prepared as follows: repurified Cohn fraction 5 human serum albumin (Squibb No. 519) was secured through the courtesy of L. Larson, Esq., Massachusetts Department of Health Biologic Laboratory, Jamaica Plain, Boston. This was dissolved in K-H bicarbonate (0.5 gram albumin/100 ml. K-H). In this concentration, the albumin contained approximately 100  $\mu$ M/L. of endogenous free fatty acids. The sodium palmitate or oleate was added so that the final solution contained the desired total quantity of free fatty acid (including the endogenous amount). Aliquots of 300 ml. of the albumin-fatty acid mixture were then dialyzed for three days against 18 L. of thoroughly gassed K-H bicarbonate at 4°C., and then filtered through two layers of number 54 Whatman filter paper. This solution was not stored for longer than one day before use. The exact stage at which the radio-active fatty acid was added, is of great importance, and will be discussed in a subsequent chapter.

The pH of the perfusate before and after perfusions with glucose, pyruvate and pyruvate with palmitate is shown in Table 2. The buffering capacity was adequate to maintain the pH at about 7.4. This is essential to avoid deterioration of cardiac force demonstrated in isolated guinea pig hearts when the pH of the perfusate fell below 7.27 (McElroy et al., 1958).



PERFUSED RAT HEART  
ANALYTICAL SEQUENCE

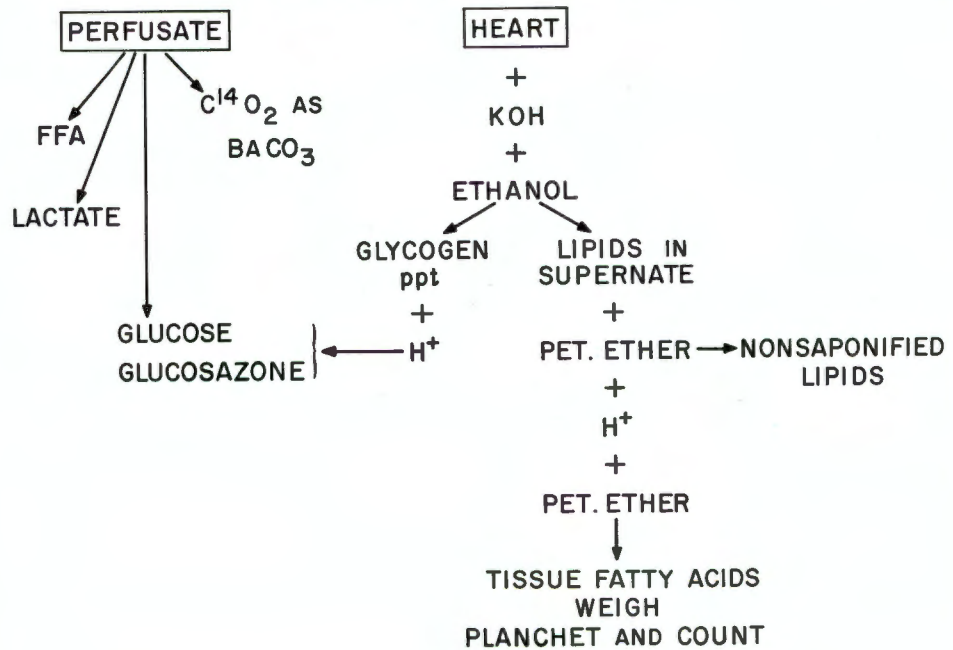


Fig 8: Schema of analytical sequence.



## (d) CARBOHYDRATE ANALYSES

The chief analytical procedures carried out on the heart and the perfusate are shown in Fig. 3. and Table 3.

Glucose and mannose were determined by Nelson's method (1944) with the Somogyi (1937) protein precipitation. Fructose was also determined by this method or by the resorcinol method (Higashi and Peters, 1950). The specific activities of  $C^{14}$ -labelled hexoses were determined by the addition of carrier and precipitation as the corresponding osazone.

Glycogen in the tissue was analysed by the method of Good, Kramer and Somogyi (1933). At the end of the perfusion the heart was rapidly incised, firmly blotted, weighed and then digested in 3 ml. hot 30% KOH within 40 seconds of the termination of the perfusion. This method of digestion gives the total glycogen, including both the easily extractable and the bound fractions (cf. Stetten and Stetten, 1960). 95% ethanol was added to precipitate the glycogen which was then hydrolyzed in acid, and the supernatant decanted and saved for tissue fatty acid determinations. An aliquot of the hydrolyzed precipitate was taken for glucose determination and glucose carrier added to the remainder, which was then precipitated as glucosazone from which the specific activity was determined. Since the glycogen content of the halves of hearts cut vertically could not be distinguished (P greater than 0.8), half hearts were used for some glycogen determinations.

The net glycogen change was defined as the difference between the final glycogen of the perfused heart and the glycogen from another series of hearts digested after pre-perfusion only. The net carbohydrate load was deduced by summing the glucose uptake and any glycogen breakdown.



**TABLE 3****ANALYTICAL PROCEDURES**

<b>For analysis of:</b>	<b>Method</b>	<b>Recovery</b>	<b>Range of Linearity</b>	<b>Standard Deviation</b>
<b>Glucose</b>	Nelson (1944) Somogyi (1937)	About 100%	50-150 ug/ml	$\pm 1.6 \%$
<b>Glycogen</b>	Good, Kramer & Somogyi (1933)	Unknown	(same as glucose)	(same as glucose)
<b>C<sup>14</sup>O<sub>2</sub></b>	Acidification & conversion to BaCO <sub>3</sub>	Unknown	Unknown	$\pm 1.5 \%$
<b>Lactate</b>	Horn & Bruns (1956) McManus (1960)	94-99 %	0-40 ug/ml	$\pm 3.8$ to 8.4 %
<b>Pyruvate</b>	Redetzki, Bloedorn & Bansi (1956) McManus (1960)	90-96 %	0-40 ug/ml	(about same as lactate)
<b>Free Fatty Acid</b>	Dole & Meinertz, (1960)	About 100%	150-1000 um/L	$\pm 4.1 \%$
<b>Tissue Fatty Acid</b>	Supernate from KOH digest of hearts hydrolyzed and extracted with pet. ether.	Same as chloroform: methanol extraction of lipids.	Unknown	Unknown



$\text{CO}_2$  specific activity was determined from the specific activity of the perfusion fluid bicarbonate by acidifying a 2 ml. aliquot of perfusate at the end of the experiment and collecting the  $\text{CO}_2$  which was precipitated and counted as barium carbonate. The total amount of  $\text{C}^{14}\text{O}_2$  was estimated from the total  $\text{CO}_2$  content of the closed unit in both gas and liquid phases. No allowance was made for  $\text{CO}_2$  production in the course of an experiment as measurements of total  $\text{CO}_2$  production (labelled and unlabelled) were not available. Instead, the total  $\text{CO}_2$  in the system was taken as 600  $\mu\text{M}$ . (375  $\mu\text{M}$ . as bicarbonate, 17  $\mu\text{M}$ . dissolved  $\text{CO}_2$ , the rest in the gaseous phase). The incorporation of glucose carbon into  $\text{C}^{14}\text{O}_2$  was given by:

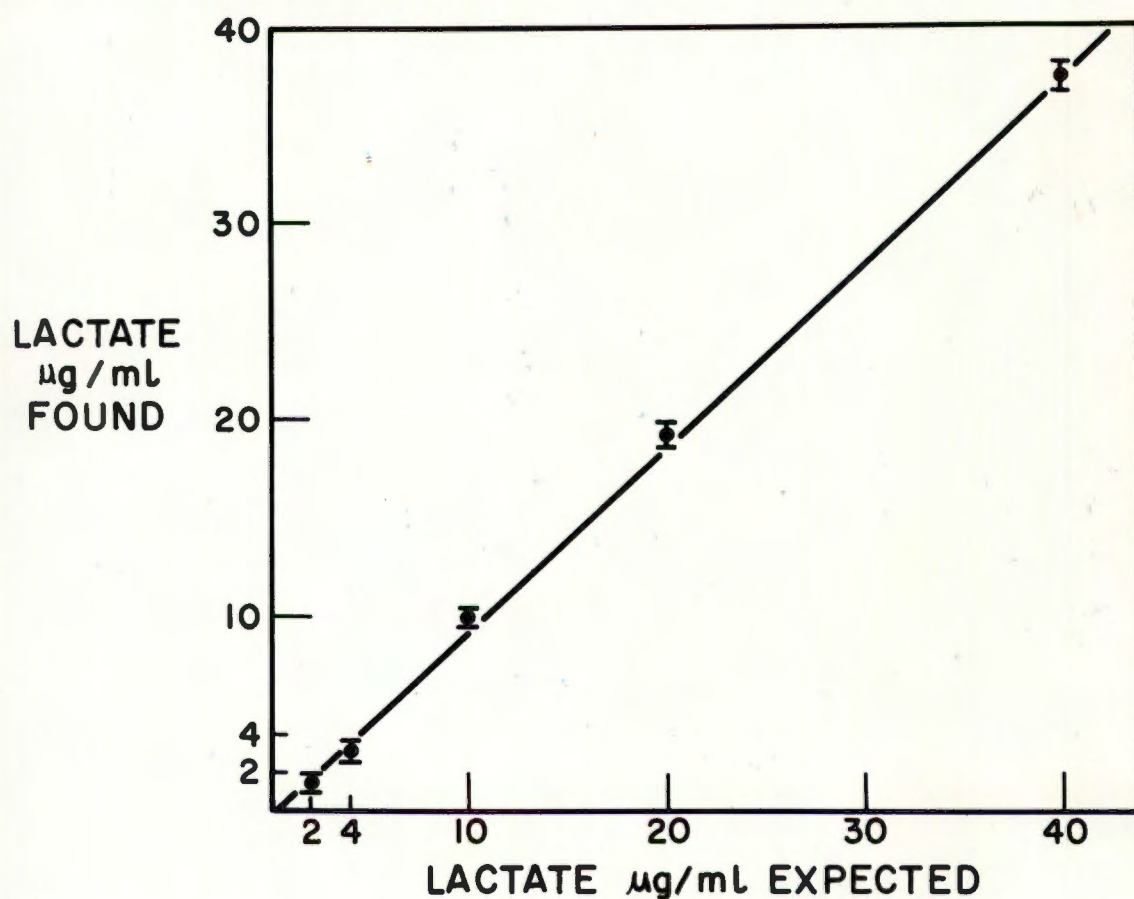
$$\frac{\text{CO}_2 \text{ Specific Activity (counts/min./}\mu\text{M.}) \times 600}{\text{Specific Activity Glucose (counts/min./}\mu\text{M.)}}$$

Lactate was determined enzymatically (cf. Horn and Bruns, 1956). 2 ml. aliquots of the perfusate underwent protein precipitation with 4 ml. of barium hydroxide (0.3 N.) and 4 ml. of zinc sulphate 5%. The supernate was diluted and 1 ml. added to 2 ml. of the following buffer: glycine 37.5 g.; sodium E.D.T.A. 1.0 g.; semicarbazide 11.0 g.; 3 N. sodium hydroxide 60 ml.; aqua ad 500 ml. (pH: 10.0). 2 mg. of D.P.N. in 0.1 ml. of water was then added, mixed and read in a Beckman D.U. at 340 m $\mu$  wavelength. Then 0.05 ml. of lactic acid dehydrogenase (2 mg. enzyme protein/ml.) was added, the contents of the cuvette mixed, and the lactate calculated stoichiometrically by the increased absorption coefficient due to reduced D.P.N. (Horecker and Kornberg, 1948). At lactate levels of 10 - 40  $\mu\text{g/ml}$ . the method was linear and the recoveries varied from 94 to 99% (Fig. 9).

Pyruvate was determined in a similar fashion, using the reverse reactions (Redetzki et al., 1956). The final perfusate was



## ENZYMATIC LACTATE: Barium -Zinc Precipitation



**Fig. 9**

The accuracy of the enzymatic lactate method. All samples were taken through Barium - Zinc precipitation.

(2 ml. sample + 4 ml. barium hydroxide + 4 ml. zinc sulphate + 15 ml. H<sub>2</sub>O).

<u>Lactate Expected</u> ug/ml.	<u>Lactate Found</u> ug/ml. se.	<u>Recovery</u> %
2	1.6 ± 0.46	80
4	3.4 ± 0.58	85
10	9.9 ± 0.41	99
20	19.3 ± 0.50	96.5
40	37.3 ± 0.71	93.5



**TABLE 4**

**F.F.A. TITRATION: WATER WASH TO REMOVE ADDED LACTATE**

Removal of lactate from 1% albumin solution containing F.F.A.  
by a single water wash of the Dole extraction mixture.

<u>Solution</u>	<u>Number</u>	<u>F.F.A.</u>
F.F.A.	4	434 $\pm$ 8*
F.F.A. + wash	4	440 $\pm$ 5
F.F.A. + Lactate 2.8 mM/L	4	463 $\pm$ 3
F.F.A. + Lactate 2.8 mM/L + wash	4	437 $\pm$ 6

\* = S. E.

F.F.A. = Free Fatty Acids



precipitated as rapidly as possible with barium hydroxide-zinc sulphate, and 0.5 ml. of the supernate added to 1.0 ml. of buffer, 1.5 ml. of distilled water, and 0.1 ml. (0.25 mg.) of D.P.N.H. (Sigma) in a cuvette. The composition of the buffer was (McManus, 1960): 0.1 N. disodiumhydrogenphosphate 500 ml.; 0.1 N. sodiumdihydrogenphosphate 500 ml.; and sodium E.D.T.A. 2 g. The pH was 6.8 to 6.9. The recovery and linearity were similar to those obtained with the lactate method (Fig. 9). When using  $C^{14}$ -labelled pyruvate, the specific activity was determined by the method of Crane and Ball (1951).

The carbohydrate recovery was the sum of the glucose equivalents of: lactate formation, glucose to  $CO_2$ , and any glycogen synthesis that might have occurred. The percent of carbohydrate load recovered was -

$$\frac{\text{carbohydrate recovery in } \mu\text{M. glucose equivalent}}{\text{carbohydrate load in } \mu\text{M. glucose equivalent}} \times 100$$

#### (e) LIPID ANALYSES

Free fatty acids (= F.F.A. = unesterified fatty acids or U.F.A. = non-esterified fatty acids or N.E.F.A.) were determined by the method of Dole and Meinertz (1960). Lactate in high concentrations is known to interfere with the determination of F.F.A. by this method. However, a single wash with 10 ml. of distilled water (pH 6.8) after the initial extraction procedure completely removed the lactate up to concentrations of 2.8 mM/L. (Table 4), and two such water washes yielded the calculated F.F.A. values in mixtures of palmitate 800  $\mu\text{M/L.}$  and acetate or pyruvate 5 mM.

To obtain the initial specific activity of radio-active fatty acids, 0.1 ml. of the fatty acid-albumin solution of known composition



was pipetted onto a very thin layer of lens paper which fitted snugly inside a planchet; the aliquot was allowed to dry and then counted. To determine the uptake of radio-activity during the perfusion, 0.1 ml. aliquots of the upper phase of the Dole extraction mixture were taken from samples before and after perfusion, and placed directly onto planchets; the aliquots were allowed to dry and then counted.

CO<sub>2</sub> specific activity was determined as for C<sup>14</sup>-labelled hexoses. Control perfusions without hearts showed that the spontaneous evolution of C<sup>14</sup>O<sub>2</sub> from palmitate-1-C<sup>14</sup> was negligible.

Tissue fatty acids were determined in two ways. First, the supernatant fluid resulting from ethanol precipitation of the KOH digest was used. The non-saponified lipids were removed with petroleum ether extraction (30-60° B.P.) and the remainder acidified to pH 1. The fatty acids were removed by three petroleum ether extractions which were pooled and purified by three aqueous washes. After drying and weighing, an aliquot was placed on a planchet and the specific activity determined.

In the second method of extraction, the heart was cut into very small pieces after the perfusion, and extracted by shaking overnight in 10 ml. chloroform-methanol 2:1. A second overnight extraction yielded no additional lipid. The extract was then made up to 20 ml. with chloroform-methanol and purified according to the Folch "salty wash" technique (Folch et al., 1957) which was repeated twice in order to fully remove radioactive contaminants (Cahill et al., 1959). The total lipids were then dried, weighed, and an aliquot placed on a planchet to determine the specific activity. The rest was saponified with an alcoholic KOH solution, and the fatty acids separated as in the first method.

In order to compare these methods of extraction, twelve hearts were cut in half vertically, after perfusion with palmitate-1-C<sup>14</sup>, and



the specific activities of the tissue fatty acids determined by the two methods. The results could not be distinguished ( $P = 0.5$ ). The KOH digest was therefore used as a more convenient way of measuring both tissue fatty acids and glycogen on the same heart.

Glyceride-glycerol specific activity was determined from the water-soluble remnant of the Folch-extraction after acidification and removal of components soluble in pet. ether. After addition of glycerol carrier, free end or alpha carbons of glycerol were oxidized with periodic acid, and the resulting formaldehyde converted to the dimesone derivative, precipitated and counted (Reeves, 1941). In calculations (cf. Cahill et al., 1959) it was assumed that 1/10 of the cardiac lipid weight was glycerol, but this approximation is of little consequence as approximately 90% of the glycerol came from the carrier.

The ketone concentration of the final perfusate was determined when indicated. The method used was a modification of that used by Werk et al. (1955).

The recovery of label from  $C^{14}$  - labelled F.F.A. was calculated as follows:

$$\text{Recovery} = \frac{\text{Total counts in } (CO_2 + \text{Tissue Fatty Acids})}{\text{Total counts disappearing from perfusate}} \times 100\%$$

#### (f) ISOTOPIC ANALYSES

All  $C^{14}$  counting was done in a windowless proportional flow counter (Nuclear Chicago). Self-absorption corrections were applied according to Karnovsky et al. (1955).



**TABLE 5****DRY/WET WEIGHT RATIOS**

<b>Condition of Hearts:</b>	<b>No. of Hearts</b>	<b>Dry weight as % wet weight <math>\pm</math> standard error</b>
<b>Directly on removal from:</b>		
(i) rats fed ad. lib.	4	$22.9 \pm 0.60$
(ii) rats fasted overnight	12	$23.8 \pm 0.12$
(iii) rats fasted 4 days	4	$23.7 \pm 0.13$
<b>After 5 min. pre-perfusion at about 60 cm. H<sub>2</sub>O</b>	4	$19.5 \pm 0.64$
<b>After similar pre-perfusion and 30 min. perfusion at 60 mm. Hg. Substrate:</b>		
(i) glucose 5 mM.	8	$17.4 \pm 0.22$
(ii) glucose 5 mM + palmitate 800 $\mu$ M/L	4	$17.6 \pm 0.41$

(mM. = mM/L)

The wet weight was usually between 800 and 1000 mg.



**TABLE 6****HEART NITROGEN CONTENT**

(Hearts from fed rats studied after perfusion with glucose 5 mM for 30 minutes at 60 mm. Hg.)

Variable:	No. of Hearts	Heart nitrogen in mg/g. wet weight ± standard error
Glucose 5mM, 38°C	4	24.4 ± 0.63
Glucose plus Ounbain 1 ug/ml.	4	24.3 ± 0.35
Glucose plus Epine- phrine 10 <sup>-7</sup> M.	4	22.1 ± 0.59
Glucose plus Nore- pinephrine 10 <sup>-7</sup> M.	4	23.5 ± 0.85
Glucose 5 mM. at 28°C.	4	22.4 ± 0.83
Perfusate gassed with N <sub>2</sub> -CO <sub>2</sub> (95:5%)	3	22.4 ± 0.18
Hearts from oxythiamine treated rats	4	23.5 ± 1.06

(mM. = mM/L ; M = M/L)



**(g) EXPRESSION OF RESULTS**

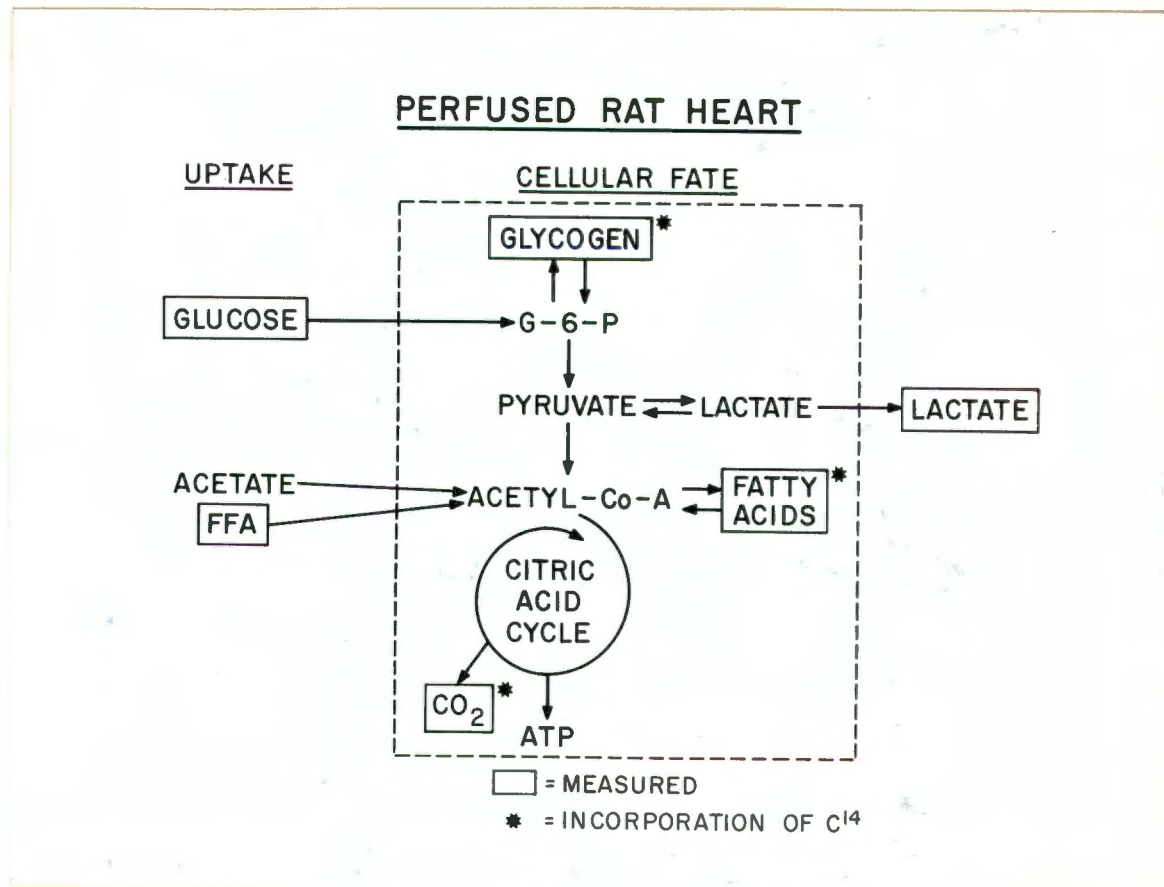
The uptake of a substrate was defined as the disappearance of that substrate from the perfusate during the perfusion period.

When using labelled hexoses, all results are expressed as  $\mu\text{M}$ . glucose equivalent (C6) per gram wet weight of heart per 30 minute perfusion. When labelled pyruvate was used, pyruvate, lactate and incorporation of label into  $\text{C}^{14}\text{O}_2$  were expressed as  $\mu\text{M}$ . C3-units per gram wet weight per 30 minute perfusion. This method of expression assumes that the proportion of water in the heart is relatively constant in all conditions. This assumption was tested in two ways. First, the dry/wet weight ratio was determined in hearts before and after pre-perfusion and perfusion (Table 5). It was concluded that feeding or fasting the donor rats did not significantly alter the dry/wet weight ratio, but that the ratio was altered by pre-perfusion and perfusion, presumably due to oedema formation. Therefore, in deducing the net glycogen change during perfusion, wet weights were corrected to correspond to dry/wet weight ratios found at the end of the perfusion unless otherwise stated. Next, the nitrogen content of the heart was determined by the micro-Kjeldahl procedure for a wide number of variables (Table 6), the perfusion in each case being for 30 minutes with glucose 5 mM/L. It was found that the wet weight at the end of the perfusion period was reasonably steady and changes greater than 10% could not be explained by changes in the nitrogen/wet weight ratio.

**(h) PREPARATION OF INSULIN**

Glucagon-free insulin was obtained through the courtesy of the

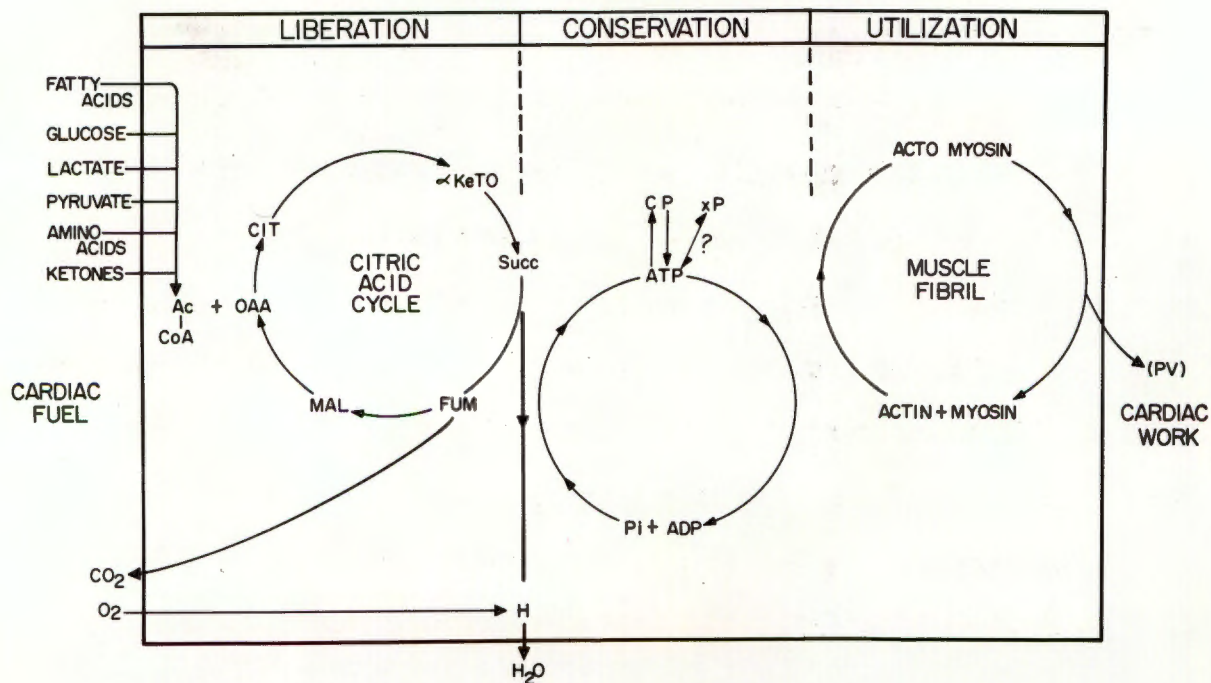




**Fig. 10** Parameters measured with methods available.



**SCHEMA OF ENERGETICS IN CARDIAC MUSCLE**  
(AFTER OLSON: N.Y.ACAD. 72: 471, 1958)



**Fig. 11** Schema of cardiac energetics.



Eli Lilly manufacturing company, and made up as an acid, concentrated solution of about 30 units/ml. This was diluted in buffer on the day of use.

#### (1) SCOPE OF THIS THESIS

The employment of the above methods allowed the measurement of the uptake of various substrates (glucose, pyruvate and free fatty acids), the incorporation of label from these substrates into  $\text{CO}_2$ , glycogen and tissue lipids, as well as the appearance of lactate in the medium (Fig. 10). When viewed in the overall context of cardiac energetics (Fig. 11), it is apparent that the techniques available allowed study of the liberation of energy rather than its conservation and utilization. The ensuing observations aim to define the uptake and fate of hexoses and free fatty acids, both individually and when present together.

With this end in view, over 100 experiments, involving about 900 perfusions, were carried out over a period of nearly two years' full-time research in the Baker Clinic Research Laboratory. The majority of perfusion and biochemical techniques have been personally performed by the author.

Preliminary reports of this work are in press (Shipp et al., 1961) and have been presented to the American Heart Association (Shipp and Opie, 1960) and the American Physiological Society (Opie et al., 1961).



### CHAPTER THREE

#### CHARACTERIZATION OF EXPERIMENTAL SYSTEM

In the preliminary investigations, the following topics were explored to test the adequacy of the perfusion system and to decide on conditions of choice for further studies.

##### (a) ADEQUACY OF OXYGENATION

###### Results:

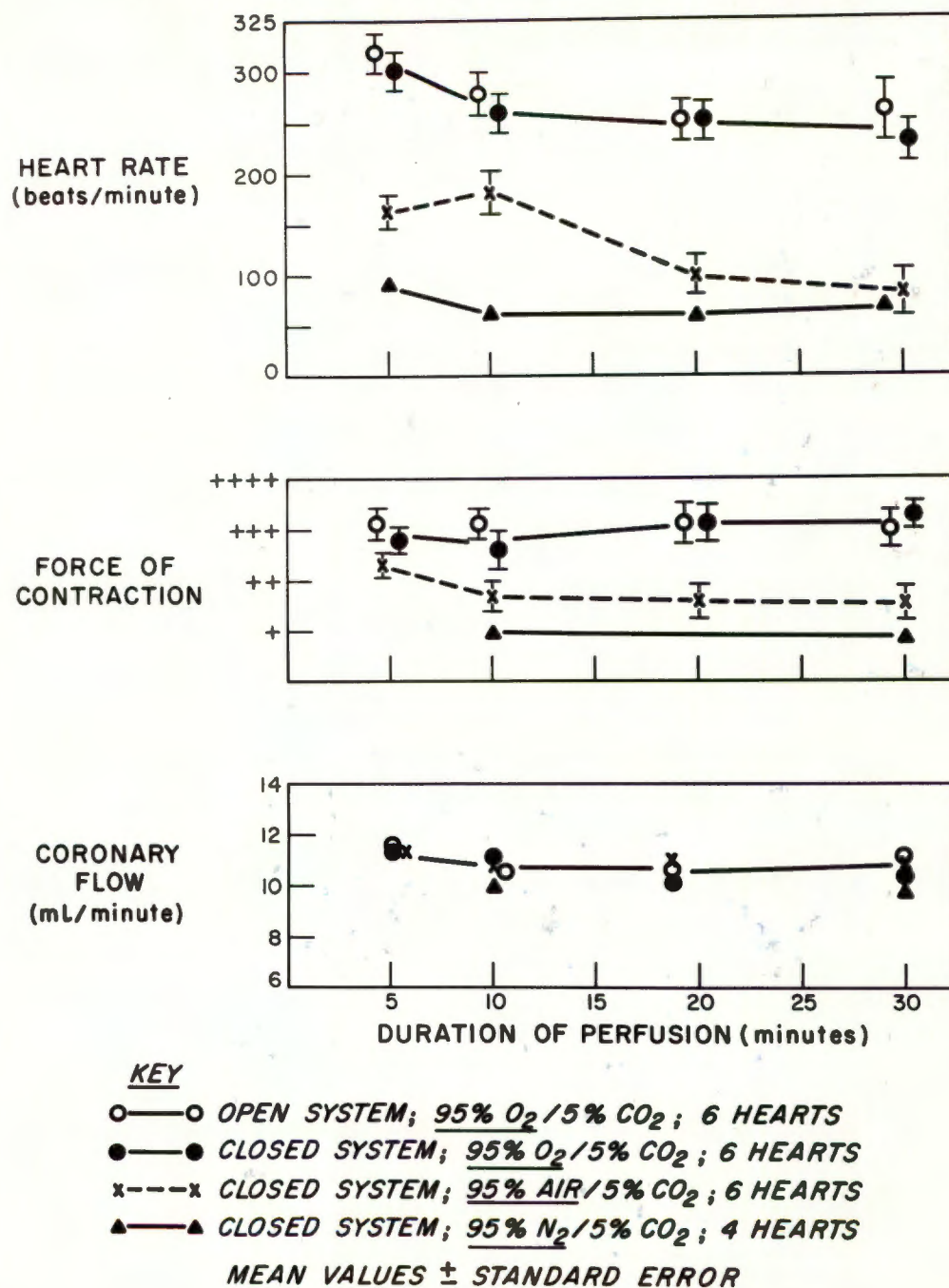
The adequacy of oxygenation of the isolated perfused rat heart was assessed by using four methods of gassing.

First, the closed system used here was oxygenated by ten minutes of vigorous bubbling of the perfusate with  $O_2-CO_2$  (95:5%) before the perfusion was started, thus yielding 15 ml. of perfusate thoroughly bubbled and about 100 ml. of  $O_2-CO_2$  (95:5%) which was closed off from the atmosphere at the start of the perfusion period. In a second method,  $O_2-CO_2$  (95:5%) was bubbled into an open perfusing system throughout the perfusion period; this is the usual system employed by other workers. Measurement of the oxygen content\* in these two methods showed average initial values of about 1.5 vols. %. Thirdly, a closed system was oxygenated with air- $CO_2$  (95:5%). Fourthly, a closed system was gassed with  $N_2-CO_2$  (95:5%). In each case the pre-perfusion was for the standard five-minute period with buffer equilibrated with  $O_2-CO_2$  (95:5%).

\*By courtesy of Dr. Richard Gorlin, Peter Bent Brigham Hospital.



# **EFFECT OF METHODS OF OXYGENATION: (I) Gross Performance of Isolated Perfused Rat Heart**



**Fig. 12** Deterioration of cardiac performance when system gassed with air or nitrogen.



# EFFECT OF METHODS OF OXYGENATION : (2)

Metabolism of Glucose -u- C<sup>14</sup> 5mM/L.

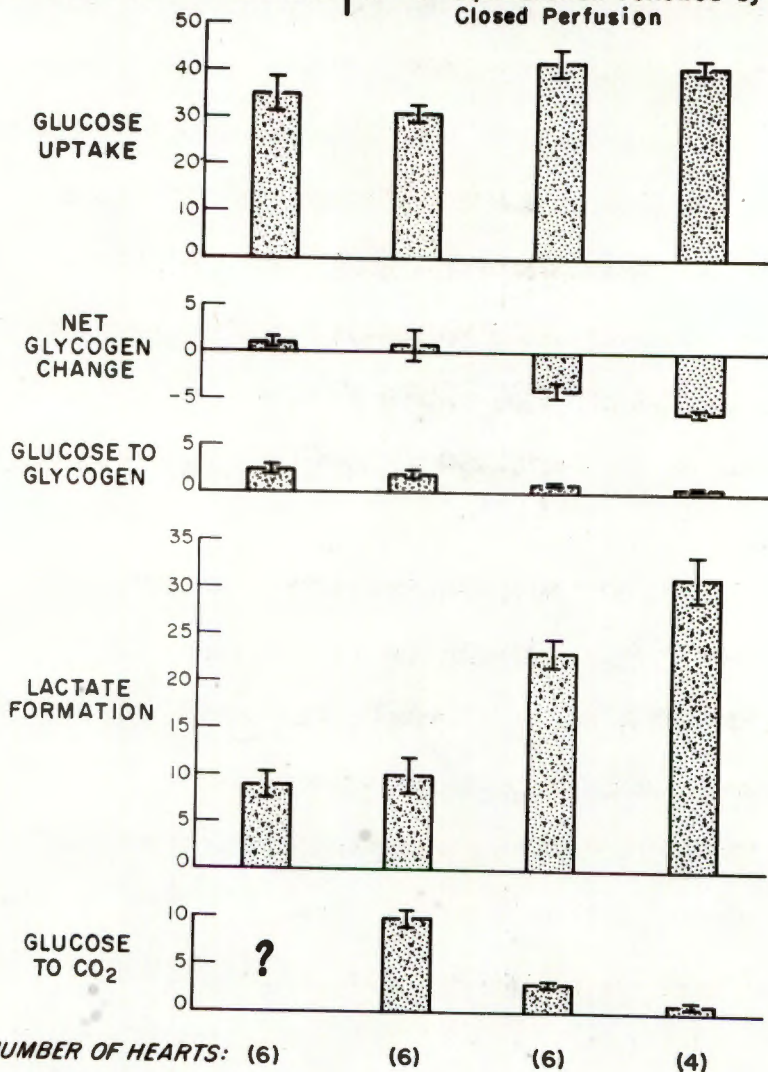
PERFUSION

GAS MIXTURE:

O <sub>2</sub> 95%	O <sub>2</sub> 95%	AIR 95%	N <sub>2</sub> 95%
CO <sub>2</sub> 5%	CO <sub>2</sub> 5%	CO <sub>2</sub> 5%	CO <sub>2</sub> 5%

METHOD OF GASSING:

Continuous Initial Equilibration Followed by Closed Perfusion



UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 min. PERFUSION

MEAN VALUES  $\pm$  STANDARD ERROR

RATS FED AD LIB

PREPERFUSION: 5 MINUTES WITH BUFFER GASSED

CONTINUOUSLY WITH 95% O<sub>2</sub> / 5% CO<sub>2</sub>

Fig. 13 Metabolic behaviour of open system (extreme left) and closed system were (second from left) similar; other systems hypoxic.



The performance of the heart and the metabolic behaviour were similar when bubbling with  $O_2$  and 5%  $CO_2$  in either closed or open system. However, when a closed system was equilibrated with air and 5%  $CO_2$  it was found that (i) the rate and force of contraction of the hearts fell; (ii) the glucose uptake increased (cf. Morgan et al., 1959); (iii) net glycogen breakdown occurred; (iv) incorporation of glucose carbon into glycogen and  $CO_2$  was diminished; and (v) lactate formation was enhanced (Figs. 12 and 13). These changes were more marked when  $O_2$  was excluded and the closed system bubbled with  $N_2$  and 5%  $CO_2$ .

#### Discussion:

Closure of the perfusion system, essential for convenient measurement of  $C^{14}O_2$ , resulted in none of the changes characteristic of hypoxia, which did, however, result from initial equilibration with room air, an unsatisfactory method of oxygenation.

The measurement of oxygen content of the perfusate before perfusion showed average values of about 1.5 vols. %, comparable to the 1.9 - 2 vols. % free oxygen in human arterial plasma (Comroe et al., 1955), but with a much higher oxygen tension of about 675 mm.Hg. ( $95 \times \frac{713}{100}$ ) as opposed to the normal human value of 110 mm.Hg. The 100 ml. gaseous phase of 95%  $O_2$  in the closed perfusion system served as a reservoir for replenishment of the  $O_2$  content of perfusate which had circulated through the heart. The total  $O_2$  content of the closed system was 95 ml. in the gaseous phase and ( $\frac{1.5}{100} \times 15$ ) ml. in the liquid phase. As the oxygen consumption of the isolated perfused rat heart is about 4.0 ml.  $O_2$ /gram wet weight/30' (Williamson, 1960), there is probably sufficient oxygen reserve.



TABLE 7

EFFECT OF PRE-PERFUSION ON SUBSEQUENT METABOLISM OF GLUCOSE-u-C<sup>14</sup> (5 mM/L)DURING PERFUSION PERIODA. The Effect of Addition of Glucose to Buffer during Pre-perfusion: Rats Fasted Overnight.

1. Pre-perfusion:				
Length		5 min.	5 min.	15 min.
Substrate		none	glucose 5 mM/L	glucose 5 mM/L
Net glycogen change		-10.4 $\pm$ 0.7	-4.7 $\pm$ 3.1	-8.7 $\pm$ 1.4
2. Perfusion:				
Glucose uptake		20.2 $\pm$ 2.6	16.3 $\pm$ 2.0	12.6 $\pm$ 2.3
Net glycogen change		-5.6 $\pm$ 1.0	-5.3 $\pm$ 1.3	-8.7 $\pm$ 1.4
Lactate formation		8.7 $\pm$ 1.8	12.0 $\pm$ 4.8	8.2 $\pm$ 4.1
Glucose to CO <sub>2</sub>		4.7 $\pm$ 0.7	2.5 $\pm$ 0.6	2.7 $\pm$ 0.6
Recovery of Carbohydrate load:		52 %	66 %	51 %

B. The Effect of Length of Pre-perfusion: Rats fed ad lib.

1. Pre-perfusion:			
Length		5 min.	15 min.
Substrate		none	none
Net glycogen change		-1.2	-5.1
2. Perfusion:			
Glucose uptake		29.9 $\pm$ 1.8	29.3 $\pm$ 1.8
Net glycogen change		-2.9 $\pm$ 1.3	0.5 $\pm$ 1.3
Lactate formation		10.3 $\pm$ 2.1	9.5 $\pm$ 2.0
Glucose to CO <sub>2</sub>		5.3 $\pm$ 0.2	4.6 $\pm$ 0.2
Recovery of Carbohydrate load:		48 %	50 %

Units -  $\mu$ M. glucose equivalent /g. wet weight/30 min.Mean values of 8 hearts  $\pm$  standard error/

No correction for changes in dry/wet wt. ratios.

- Note: (i) Pre-perfusion with glucose added to buffer reduces glucose uptake and glucose to CO<sub>2</sub>
- (ii) Highest glucose uptake and least glycogen breakdown in heart from rats fed ad lib.
- (iii) 5 and 15 minute pre-perfusions have similar effects.



In all subsequently reported experiments the closed system with 95%  $O_2$  and 5%  $CO_2$  was used. Hearts with unsatisfactory rate, force or coronary flow were discarded.

#### (b) TYPE AND LENGTH OF PRE-PERFUSION

##### Results:

Experiments with two lengths of pre-perfusion (5 and 15 minutes) with both plain buffer and buffer containing 5 mM glucose were carried out to demonstrate the effect of pre-perfusion on subsequent metabolism of glucose- $u-C^{14}$  in hearts from rats fasted overnight (Table 7A).

The addition of glucose to the buffer during pre-perfusion affected the subsequent perfusion in that glucose uptake and  $CO_2$  formation were reduced.

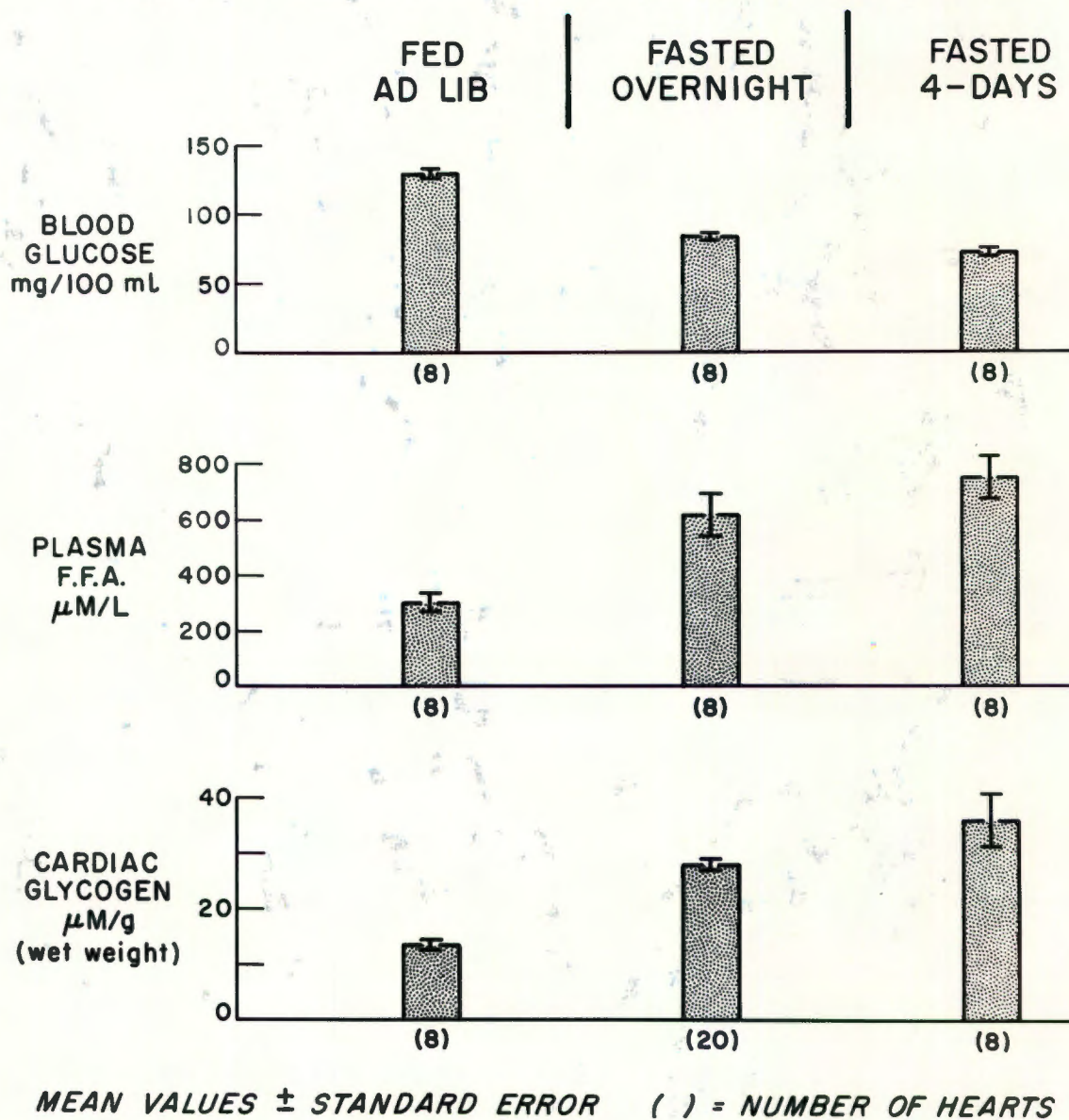
A further experiment compared the effect of 5 and 15 minutes pre-perfusion with buffer only, using hearts from rats fed ad lib. (Table 7B). The variation in the length of the pre-perfusion did not affect glucose metabolism in the perfusion. The glucose uptake was, however, greater in hearts from rats fed ad lib. than in hearts from rats fasted overnight.

##### Discussion:

The length and type of pre-perfusion were important since they influenced the subsequent handling of glucose during the perfusion period. Using hearts from rats fasted overnight, pre-perfusion with plain buffer was the method of choice since it resulted in the highest glucose uptake and  $CO_2$  formation in the subsequent perfusion. Because the pre-perfusion was substrate-free, there was decided glycogen breakdown during this



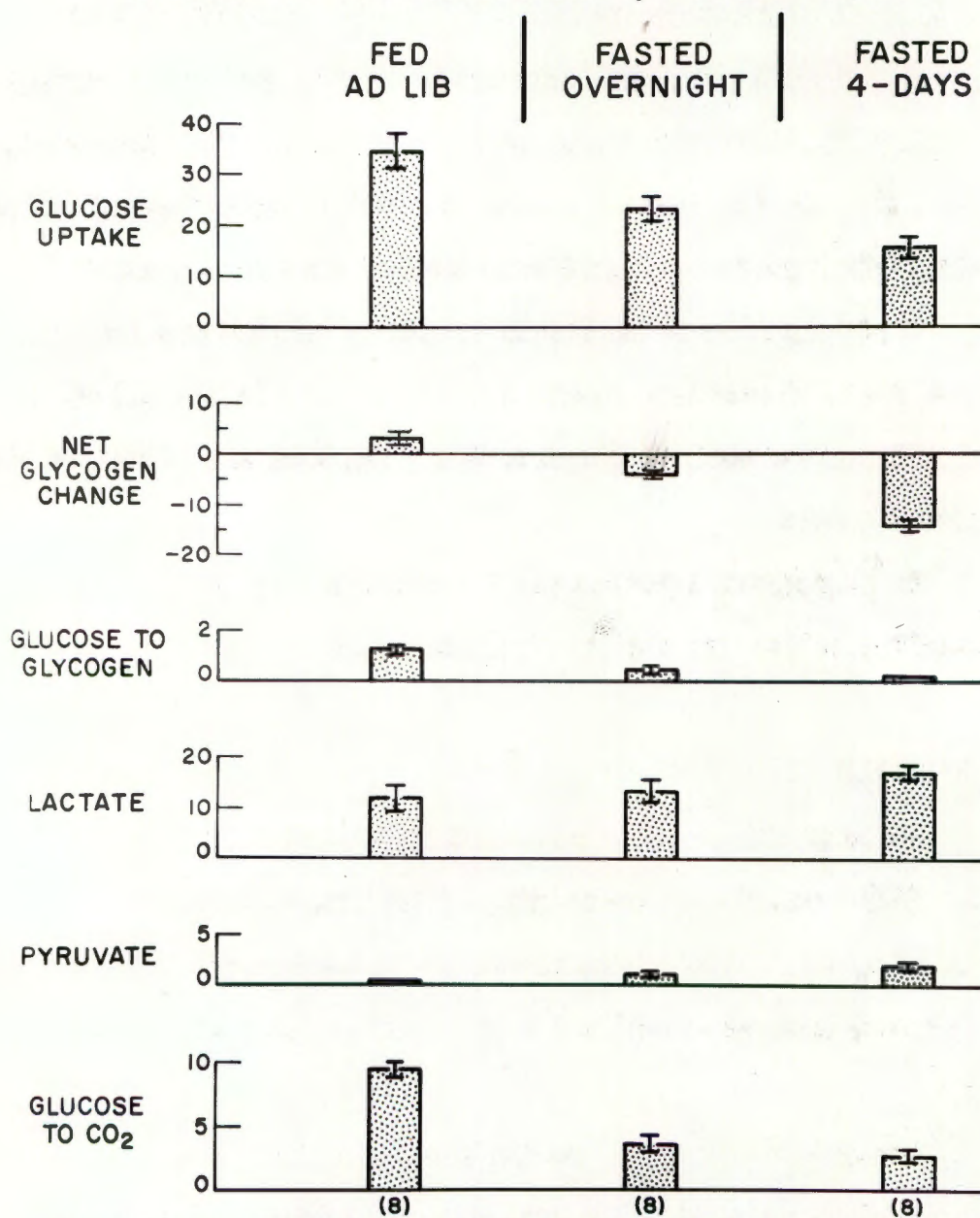
**NUTRITIONAL STATE OF RAT: (1) Blood Sugar,  
Plasma F.F.A., and Cardiac Glycogen on Decapitation**



**Fig. 14** Blood sugar fell during fasting, but plasma free fatty acids and cardiac glycogen rose.



**NUTRITIONAL STATE OF RAT: (2) Effect on  
Glucose - u - C<sup>14</sup> Metabolism by Perfused Heart**



UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 min. PERFUSION

MEAN VALUES  $\pm$  STANDARD ERROR ( ) = NUMBER OF HEARTS

Glucose concentration = 5 mM/L.

**Fig. 15** Fasting donor rats depressed myocardial glucose handling during perfusion.



period, an advantage because it minimized the possible effects of endogenous glycolysis on the glucose uptake during the actual perfusion period. When hearts from fed rats were used, the initial cardiac glycogen was lower (cf. Fig. 14; Adrouny & Russell, 1956), and an initial period of substrate-free perfusion would have been of less importance.

There appeared to be little to choose between pre-perfusion periods of 5 and 15 minutes, and as 5 minutes could remove 98% of an extracellular marker such as sorbitol-1-C<sup>14</sup> (Park et al., 1959), 5 minutes was deemed adequate.

In subsequent experiments, 5 minutes of pre-perfusion with substrate-free buffer was the standard procedure.

#### (c) NUTRITIONAL STATE OF DONOR RATS

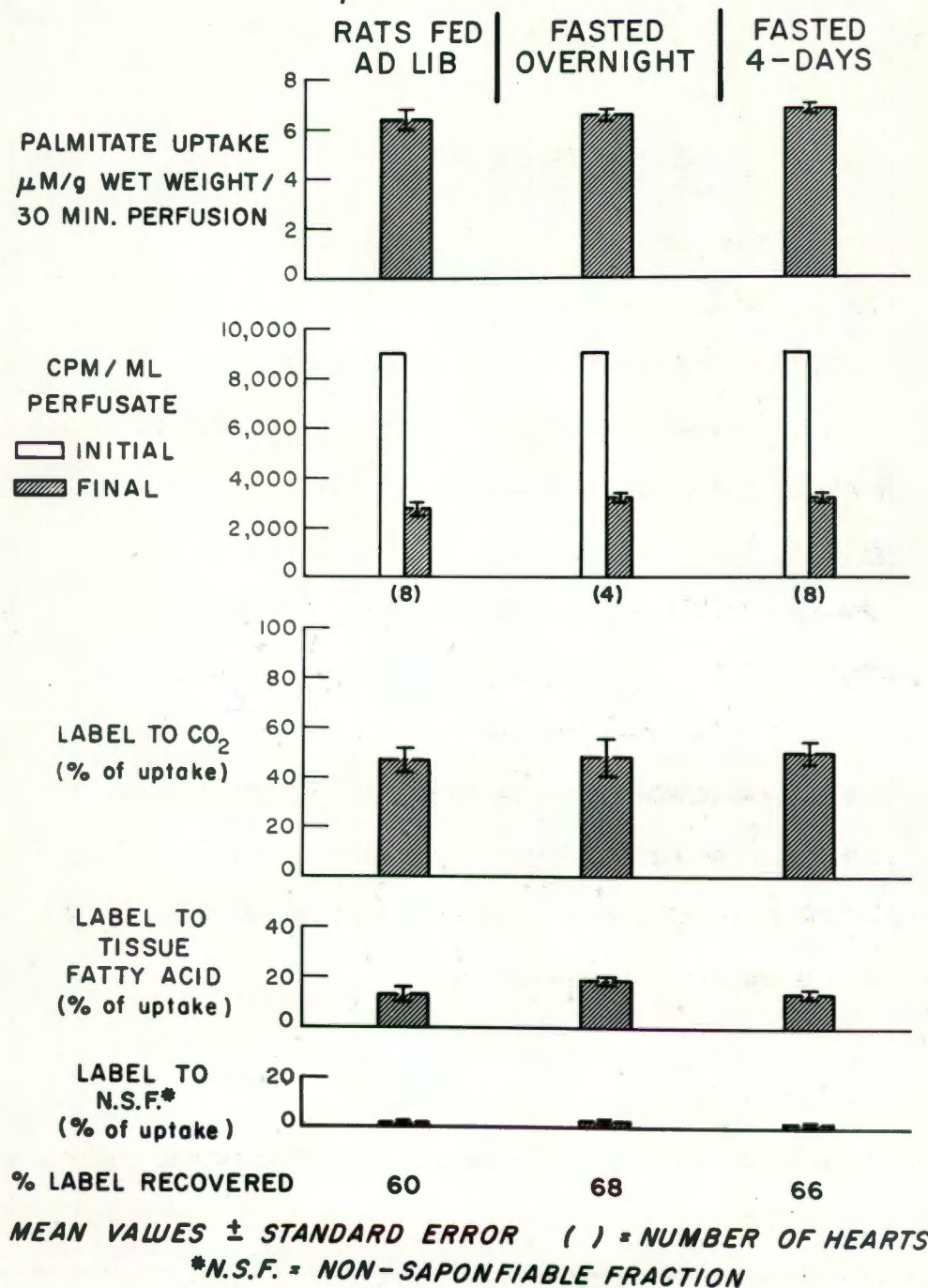
Three groups of eight rats each were studied: (i) rats fed ad lib., (ii) rats fasted overnight, (iii) rats fasted for four days. The first group was allowed free access to Purina rat pellets (Ralston Purina Company) and water, while the other groups were allowed only water.

#### Results:

The effect of fasting on the blood glucose, plasma F.F.A. and cardiac glycogen at the time the rats were decapitated is shown in Fig. 14. The following progressive changes occurred: a fall in blood glucose, a rise in plasma F.F.A. and an increase in cardiac glycogen. Fasting the donor rats for four days resulted in a decrease in the glucose uptake of the isolated perfused heart by about half (Fig. 15). Fasting also increased glycogenolysis in the perfusion period, enhanced lactate production and diminished incorporation of glucose carbon into CO<sub>2</sub> and



**NUTRITIONAL STATE OF RAT: (3) Effect on  
Palmitate- $1-C^{14}$  (750  $\mu$ M/L) Metabolism by Perfused Heart**



**Fig. 16** Fasting did not influence myocardial F.F.A. metabolism during perfusion.



glycogen. The changes in pyruvate production, as shown in Fig. 15, were:

<u>Pyruvate production</u> uM. C6/g.w.w./30 min.	
Rats fed ad lib.	$0.3 \pm 0.04$
Rats fasted overnight	$1.0 \pm 0.02$
Rats fasted 4 days	$1.9 \pm 0.30$

In contrast to the above findings, the metabolism of palmitate- $1\text{-C}^{14}$  (750 uM/L.) was similar in the three groups (Fig. 16).

#### Discussion:

Because fasting caused the glucose uptake and fate to undergo marked changes (as will be discussed in Chapter 4), further studies of glucose metabolism used both fed and fasted rats whenever possible. The six-fold rise in perfusate pyruvate levels after the four-day fast will be commented on in the next chapter. When studying F.F.A. metabolism, the rat's nutritional status was considered to be of less consequence, and rats were generally fasted overnight.

#### (d) RECOVERY OF LABEL

When using radio-active hexoses, the recovery of label could not be adequately calculated because techniques to separate and precipitate labelled lactate were not available. Instead, the percentage recovery of the carbohydrate load (as defined on p. 11) was used. The usual recovery varied from 60 to 80% (Tables 8 and 9) but occasionally it was only about 50% (Table 7). These recoveries indicated that the greater part of the carbohydrate load could be accounted for and that the major fates were those measured, i.e. glycogen, lactate and  $\text{CO}_2$ .



TABLE 8

RECOVERY OF CARBOHYDRATE LOAD:

PERFUSION WITH GLUCOSE 5mM

Condition:	Fig.:	Recovery of Carbohydrate load:
Control: O <sub>2</sub> -CO <sub>2</sub>	13	66 %
Air-CO <sub>2</sub>	13	60 %
N <sub>2</sub> -CO <sub>2</sub>	13	68 %
Control, fed ad. lib.	15	72 %
Fasted overnight	15	65 %
Fasted 4 days	15	65 %

Sample calculation (glucose 5 mM. O<sub>2</sub>-CO<sub>2</sub>):  $\mu\text{M C6/g.w.w./30 min.}$

A. Carbohydrate load:

Glucose uptake (30.7) + glycogen breakdown (0)

B. Carbohydrate Recovery:

Net glycogen synthesis (0.7) + lactate as C6 (9.9)  
+ glucose to CO<sub>2</sub> (9.7)

C. % Recovery of Carbohydrate load:

$$\frac{0.7 + 9.9 + 9.7}{30.7} \times 100 = 66\%$$

(mM. = mM/L.)



**TABLE 9**

**RECOVERY OF CARBOHYDRATE LOAD AT VARIOUS GLUCOSE CONCENTRATIONS**

**RATS FED AD. LIB.**

<b>Glucose conc. mM</b>	<b>Carbohydrate load Recovered</b>
<b>1.25</b>	<b>64 %</b>
<b>2.5</b>	<b>87 %</b>
<b>5.0</b>	<b>71 %</b>
<b>10.0</b>	<b>72 %</b>
<b>20.0</b>	<b>50 %</b>
<b>40.0</b>	<b>36 %</b>



when using radio-active palmitate, usually about 60 - 70% of the label was recovered (Fig. 16), again indicating that the major fates of the label had been traced to tissue fatty acids and  $\text{CO}_2$ .

#### (e) CONCLUSIONS

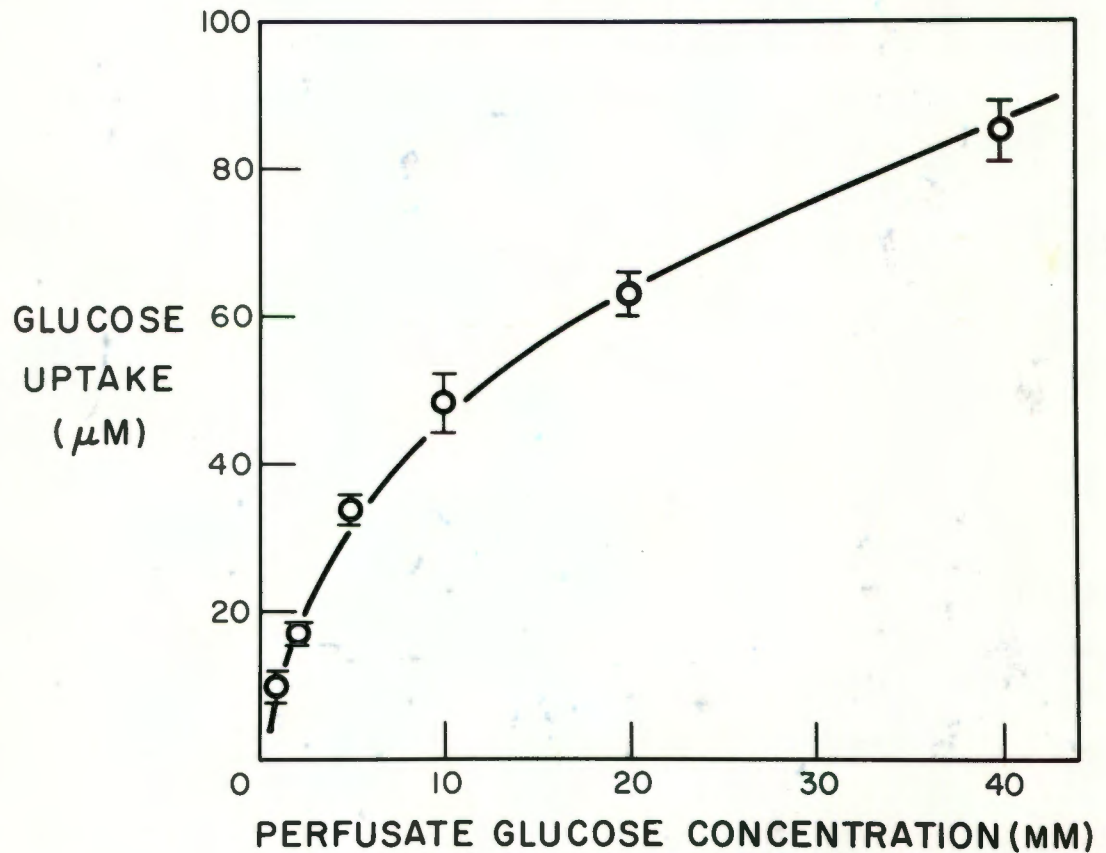
These initial studies established that:

- (i) The closed system was suitable to study the intermediary myocardial metabolism of the isolated perfused rat heart with radio-isotopes; and that oxygenation was as good as with systems employing continuous gassing;
- (ii) Pre-perfusion for 5 minutes with substrate-free buffer was a satisfactory method of initially washing out the heart;
- (iii) Fasting of donor rats affected glucose but not palmitate metabolism by the isolated rat heart;
- (iv) The major fates of glucose and palmitate metabolism were studied.



**GLUCOSE METABOLISM: (I) Glucose Uptake**  
**Related to Perfusate Glucose**

*RATS FED AD LIB*

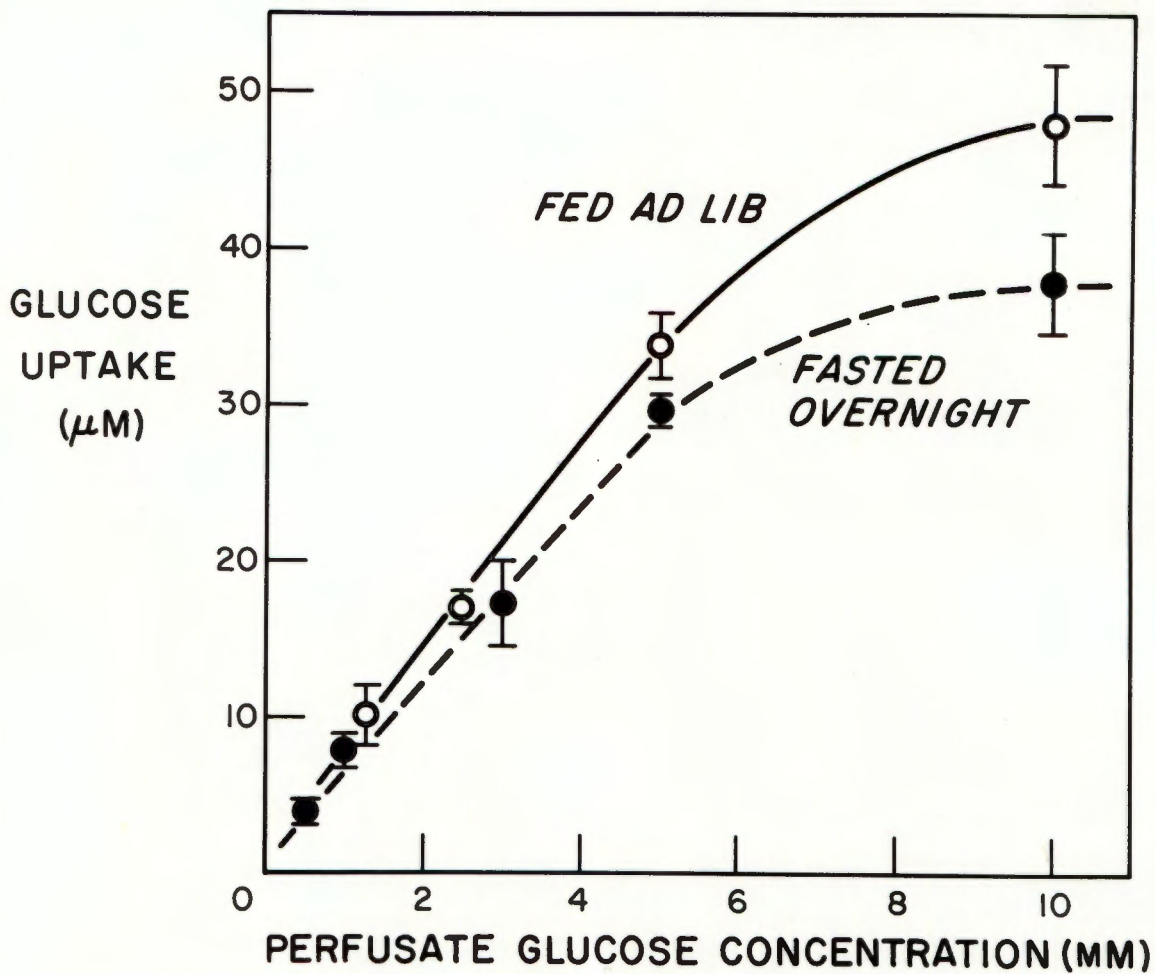


*UPTAKE IN  $\mu\text{M}$  GLUCOSE/g WET WEIGHT/30 MIN.  
MEAN VALUES OF 4 OR MORE HEARTS  $\pm$  STANDARD ERROR*

**Fig. 17** In the fed state, glucose uptake did not reach a maximum with increasing perfusing concentrations.



## GLUCOSE METABOLISM: (2) Glucose Uptake



UPTAKE IN  $\mu\text{M}$  GLUCOSE/g WET WEIGHT/30 MIN.  
MEAN VALUES OF 4 HEARTS  $\pm$  STANDARD ERROR

**Fig. 18** Glucose uptake was lower in hearts from rats fasted overnight.



## CHAPTER FOUR

### HEXOSE METABOLISM

In the intact dog and in men, substrate uptake by the myocardium is generally related to the arterial level of that substrate (cf. Ding, 1954). In the isolated perfused rat heart, glucose uptake has been related to the glucose concentration in the perfusate by Morgan and co-workers (1961), but they only used fasted rats, and did not trace the fate of the glucose uptake. Furthermore, the myocardial handling of other hexoses is largely undefined.

An investigation of hexose handling by perfused rat heart was therefore undertaken to delineate: (a) the effect of various perfusate glucose concentrations on the uptake and fate of glucose- $u\text{-C}^{14}$  in hearts from fed and fasted rats; (b) the effect of insulin on the glucose uptake and fate; and (c) the handling of mannose- $u\text{-C}^{14}$  and fructose- $u\text{-C}^{14}$ .

#### (a) UPTAKE AND FATE OF GLUCOSE

Results: The glucose concentration in the perfusate was varied from 0.5 to 40 mM. and plotted against the resulting uptake (Figs. 17 and 18).

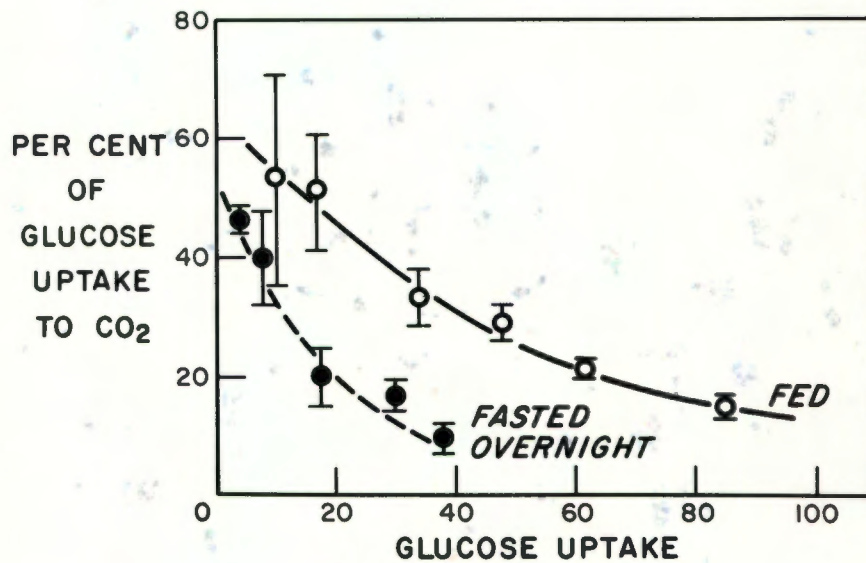
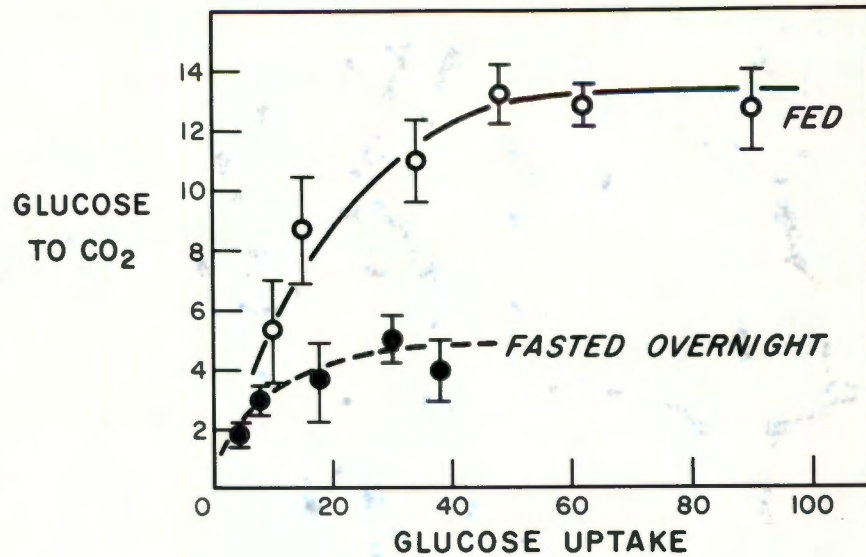
The incorporation of label from glucose into  $\text{C}^{14}\text{O}_2$  and glycogen, definitely reached a plateau with increasing glucose uptakes (Fig. 19), while net glycogen change and lactate production reached a less definite plateau (Figs. 20 and 21). The recovery of the carbohydrate load was least at the highest glucose uptakes (Tables 9 and 10).

#### Discussion:

The glucose uptake was greater in hearts from fed rats, especially at higher perfusate concentrations. The detectable uptake even



**GLUCOSE- $u$ - $C^{14}$  METABOLISM: (3)  $CO_2$  Production  
Related to Glucose Uptake**

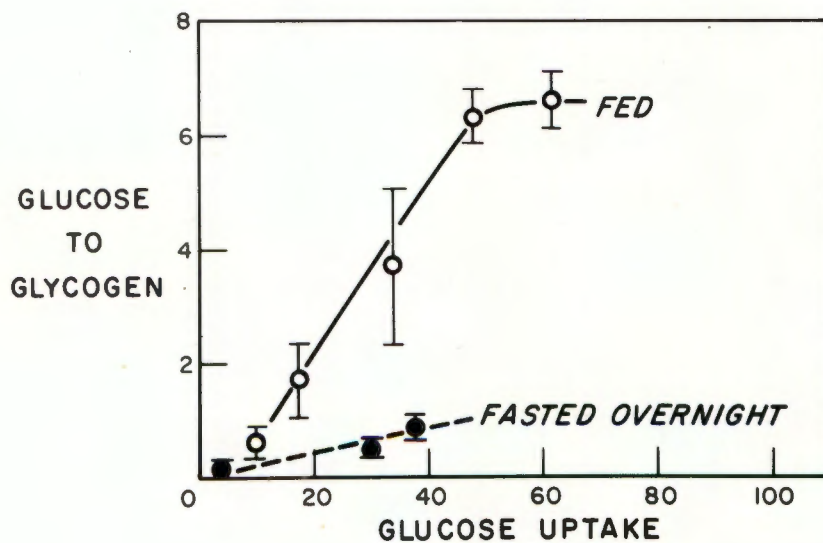
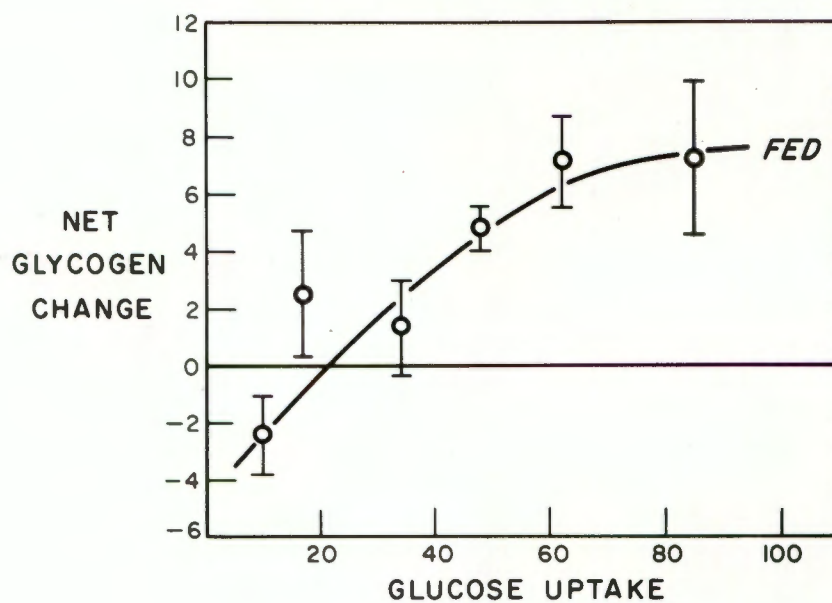


UNITS =  $\mu M$  GLUCOSE EQUIVALENT/g WET WEIGHT/30 MIN.  
MEAN VALUES OF 4 OR MORE HEARTS  $\pm$  STANDARD ERROR

**Fig. 19** Greater  $CO_2$  production from glucose in fed state.



**GLUCOSE-u-C<sup>14</sup> METABOLISM: (4) Glycogen  
Related to Glucose Uptake**



UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 MIN.  
MEAN VALUES OF 4 OR MORE HEARTS  $\pm$  STANDARD ERROR

**Fig. 20** Greater incorporation of glucose label into glycogen in the fed state.



at very low concentrations agrees with the findings of Morgan et al., (1961) in the perfused rat heart and with those of Olson and Platnick (1959) using heart slices.

The glucose threshold demonstrated in vivo in both dog and man (Bing et al., 1953; Goodale and Hackel, 1953; Goodale et al., 1959) was not observed. The threshold in vivo may be more apparent than real. It is difficult to obtain really low glucose levels in vivo without introducing factors such as fasting or insulin which may in themselves affect glucose uptake by other mechanisms.

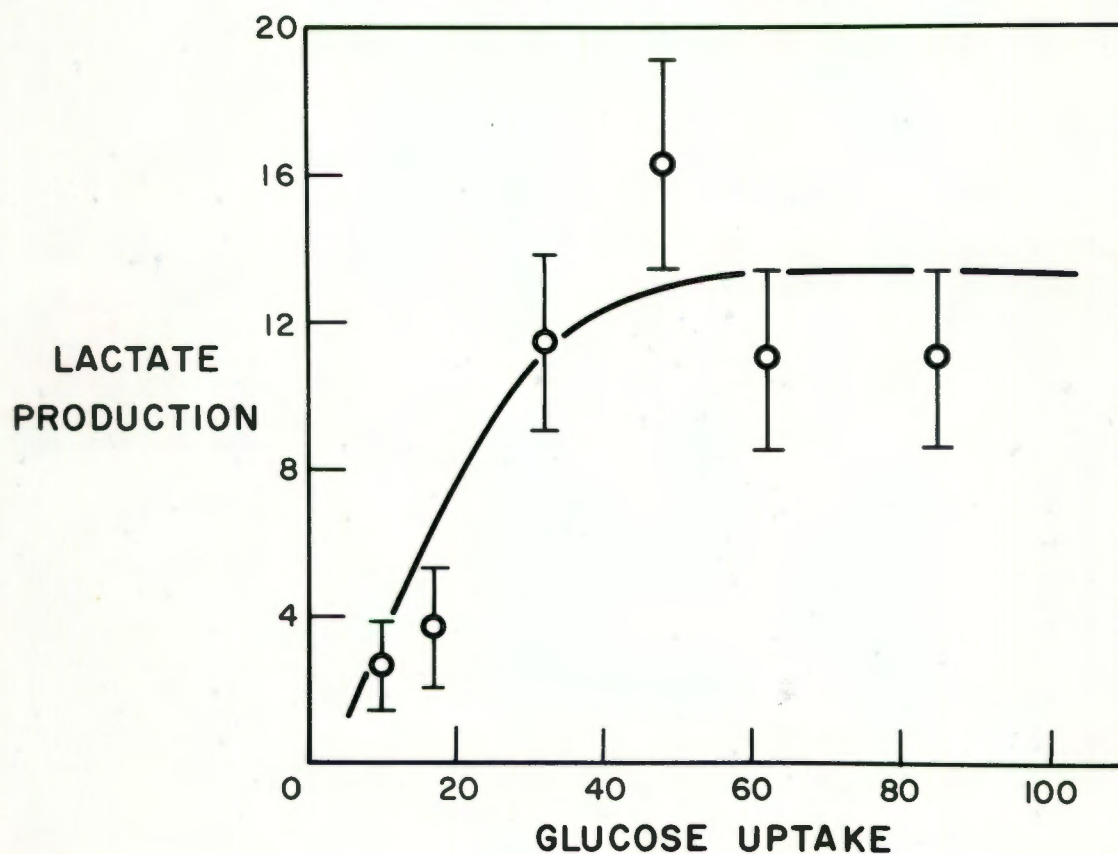
The fate of the glucose taken up was related to the magnitude of the uptake and therefore also to the perfusate glucose concentration. At low glucose uptakes a greater percentage of the uptake was recovered as  $C^{14}O_2$ , suggesting preferential use of glucose uptake for oxidative purposes at these low levels, and at such concentrations an increase in glucose uptake increased the incorporation of glucose label into  $C^{14}O_2$ . At higher uptake glucose label incorporation into  $C^{14}O_2$  reached a plateau. This strongly suggested that the rate of glucose uptake exceeded the oxidative capacity of the system. If, in addition, a definite plateau was indeed reached in formation of lactate, net glycogen, and incorporation of label from glucose into glycogen, then the suggestion is that the rate of uptake exceeded the rate of utilization. This would agree with the diminished recovery of carbohydrate as the glucose uptake increased. (Table 9).

The effect of fasting the donor rats overnight was to cause a greater diminution of the incorporation of glucose label into  $C^{14}O_2$  and glycogen than could be accounted for by the decreased glucose uptake



# **GLUCOSE METABOLISM: (5) Lactate Production Related to Glucose Uptake**

*RATS FED AD LIB*



*UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 MIN.  
MEAN VALUES OF 4 OR MORE HEARTS  $\pm$  STANDARD ERROR*

**Fig. 21** Lactate production <sup>may have</sup> reached a plateau with increasing glucose uptake.



(cf. Figs. 18 - 20).

One suggestion is that glucose metabolism was impaired at some state after transport across the cell membrane but before formation of glucose-6-phosphate. This would concur with the finding of Kipnis et al., (1959), that phosphorylation in the rat diaphragm is depressed by fasting; the defect could, however, only be elicited by a high plasma glucose level (about 20 mM/L) together with insulin.

Another factor that could possibly explain diminished incorporation of glucose label into both  $C^{14}O_2$  and glycogen, would be the greater glycogenolysis during perfusion of hearts from fasted rats. This glycogenolysis must be due to some abnormality introduced during the perfusion because in vivo the cardiac glycogen increases rather than decreases with fasting (Fig. 14; Evans 1933). Glycogenolysis could have lead to an accumulation of glycolytic intermediaries and a resulting inhibition of glucose uptake. Similar mechanisms were invoked by Patterson and Sterling (1913) in studies on the effect of epinephrine on the heart. The more sophisticated measurements of Kipnis et al. (1959) have strongly suggested that, after epinephrine administration, glucose entry into diaphragm is controlled by an accumulation of its phosphate ester. In addition to affecting glucose uptake, glycogenolysis might apparently decrease  $C^{14}O_2$  formation by diluting labelled  $CO_2$  precursors.

The measured incorporation of glucose carbon into glycogen at the end of the perfusion must depend on a balance of labelled glucose carbon entering and leaving glycogen. Glycogenolysis could decrease the incorporation of glucose carbon into glycogen simply by promoting the egress of labelled carbon from glycogen without necessarily influencing the distinctly



TABLE 10

EFFECT OF INSULIN ON GLUCOSE-u-C<sup>14</sup> METABOLISM

Gluc. Conc. uM.	Rats fed or fasted	Insulin	Glucose uptake	Net Glycogen	Glucose to Glycogen		Lactate	Recovery
1.25	Fed	none	10.0 $\pm$ 1.2	-2.4 $\pm$ 1.4	0.6 $\pm$ 0.2	5.3 $\pm$ 1.8	2.6 $\pm$ 1.3	64%
(Exp. 100)	"	2 mu/ml	14.2 $\pm$ 0.6	-1.3 $\pm$ 1.2	2.4 $\pm$ 0.4	7.4 $\pm$ 0.8	2.0 $\pm$ 1.0	61%
2.5	Fed	none	17.2 $\pm$ 0.7	2.5 $\pm$ 2.3	1.7 $\pm$ 0.7	8.7 $\pm$ 1.8	3.8 $\pm$ 1.8	87%
(Exp. 100)	"	2 mu/ml	27.9 $\pm$ 3.3	5.9 $\pm$ 1.3	4.3 $\pm$ 1.3	10.2 $\pm$ 1.3	3.4 $\pm$ 0.7	70%
5.0	Fed	none	33.9 $\pm$ 1.8	1.4 $\pm$ 1.7	3.7 $\pm$ 1.4	11.0 $\pm$ 1.4	11.5 $\pm$ 2.4	71%
(Exp. 100)	"	2 mu/ml	42.6 $\pm$ 1.8	6.3 $\pm$ 1.6	5.7 $\pm$ 2.0	10.6 $\pm$ 1.0	8.2 $\pm$ 2.0	59%
5.0	Fed	none	23.2 $\pm$ 2.3	2.0 $\pm$ 0.9	1.8 $\pm$ 0.3	10.0 $\pm$ 0.8	9.3 $\pm$ 1.8	92%
(Exp. 94)	"	2 mu/ml	32.3 $\pm$ 1.8	4.0 $\pm$ 2.4	2.2 $\pm$ 0.9	9.9 $\pm$ 0.3	15.6 $\pm$ 1.8	91%
20	Fed	none	44.2 $\pm$ 3.3	5.1 $\pm$ 2.8	2.0 $\pm$ 0.7	11.8 $\pm$ 1.7	4.6 $\pm$ 1.1	49%
(Exp. 103)	"	2 mu/ml	44.4 $\pm$ 6.3	11.3 $\pm$ 0.7	9.4 $\pm$ 0.8	12.4 $\pm$ 1.7	4.7 $\pm$ 0.7	64%
	"	100 "	64.6 $\pm$ 7.4	8.8 $\pm$ 1.5	9.0 $\pm$ 1.3	15.8 $\pm$ 1.2	16.7 $\pm$ 7.4	64%
20	Fasted overnight	none	30.7 $\pm$ 8.1	1.0 $\pm$ 0.5	1.2 $\pm$ 0.2	4.7 $\pm$ 1.7	9.4 $\pm$ 2.9	49%
(Exp. 103)	"	2 mu/ml	42.4 $\pm$ 7.9	6.2 $\pm$ 1.4	2.2 $\pm$ 0.3	5.3 $\pm$ 2.2	9.7 $\pm$ 1.9	50%
	"	100 "	48.6 $\pm$ 1.3	3.8 $\pm$ 2.0	2.2 $\pm$ 0.5	8.1 $\pm$ 1.3	17.8 $\pm$ 5.6	55%

Units= uM glucose equivalent/g. wet weight/30 min.  
Mean values of 4 hearts  $\pm$  standard error.  
Recovery = Carbohydrate recovery.

Note following effects of insulin:

- (i) increase of 50% in glucose uptake
- (ii) consistent rise in glycogen
- (iii) slight increase in CO<sub>2</sub>
- (iv) variable effect on lactate
- (v) effect found in both fed and fasted rats



different process of glycogen synthesis.

Defective myocardial carbohydrate oxidation during fasting has been inferred by Goodale and Hackel (1953) who worked with intact dogs. Decreased  $C^{14}O_2$  production from glucose by hearts from fasted rats could be due to a similar depression of oxidative mechanisms for carbohydrate. Such a defect cannot reside in the Krebs cycle because F.F.A. oxidation is unimpaired in fasting (Fig. 16). Prolonged fasting also caused a six-fold accumulation of pyruvate in the perfusate (cf. p. 20). This cannot be explained on the basis of impaired glucose phosphorylation and increased glycolysis, and suggests that fasting either depresses pyruvate oxidation at its entry into the Krebs cycle or depresses its conversion to lactate.

#### (b) EFFECT OF INSULIN

##### Results:

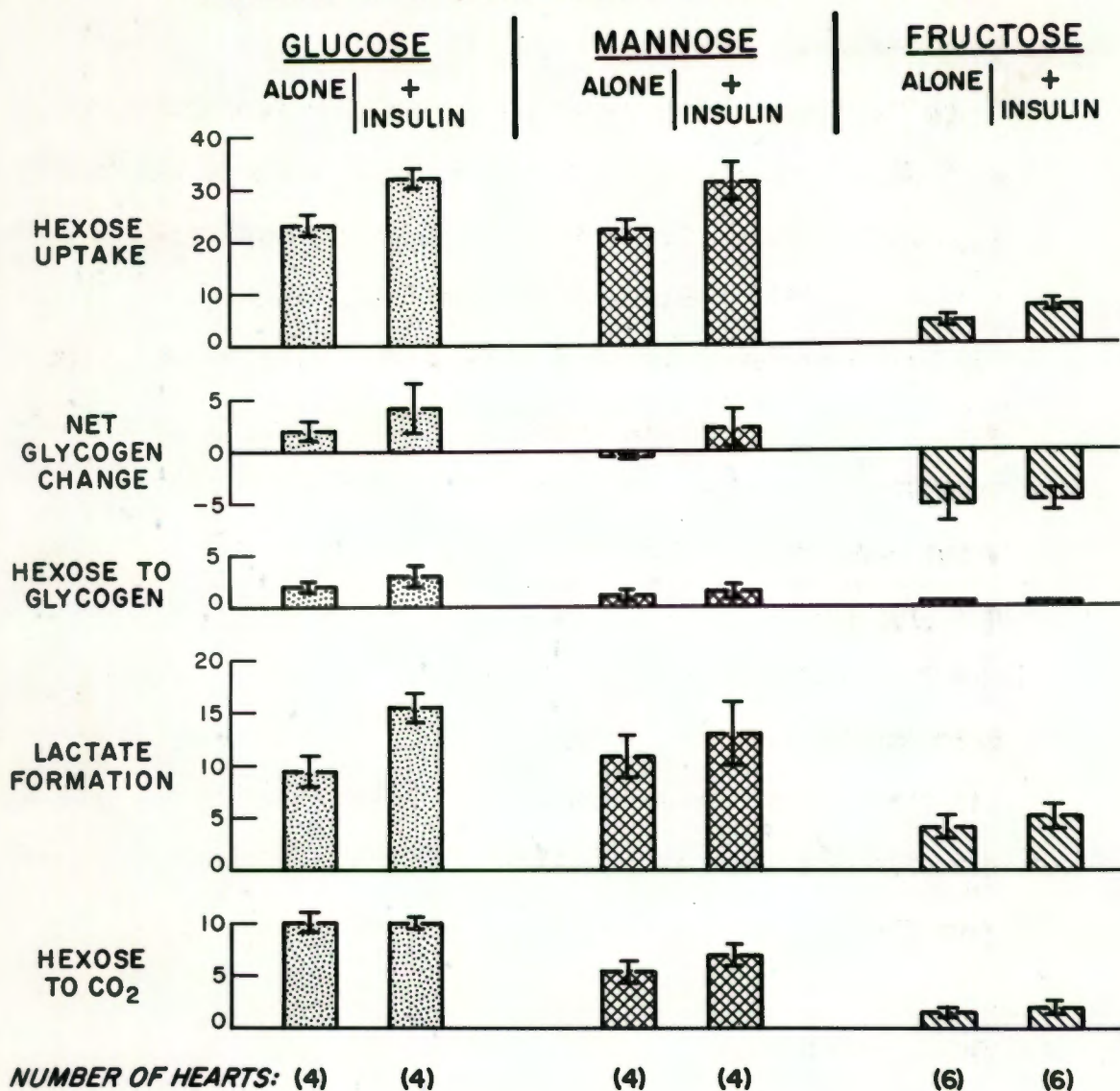
The effect of insulin on the uptake and fate of glucose- $u-C^{14}$  was studied with the glucose concentration of the perfusate varying from 1.25 to 20 mM., with the results shown in Table 10. The values at 5 mM. exemplified the occasional variations in glucose uptake that occurred. Results were therefore always compared with controls done on the same day.

##### Discussion:

One effect of insulin was to increase the cardiac uptake of glucose by about 50%. This was a lesser increase than that reported by other workers (e.g. Bleehen & Fisher, 1954; Park et al., 1959) but, as has been pointed out, the increase of glucose uptake with insulin depends very much on minor and not too obvious variations in technique, and different workers in the same laboratory have reported increases varying from two-fold to eight-fold (Williamson, 1960). This increased uptake, stimulated



**HEXOSE METABOLISM: (1) Comparison of Glucose-u-C<sup>14</sup>, Mannose-u-C<sup>14</sup>, and Fructose-u-C<sup>14</sup> 5mM/L**



RATS FED AD LIB INSULIN =  $2 \times 10^{-3}$  u/ML

UNITS =  $\mu$ M HEXOSE EQUIVALENT/g WET WEIGHT/30 min. PERFUSION

MEAN VALUES  $\pm$  STANDARD ERROR

**Fig. 22** Hexose handling: mannose similar to glucose; fructose uptake slight; all insulin-sensitive.



by insulin, was recovered as glycogen or  $\text{CO}_2$ , but only occasionally was lactate increased.

In the isolated diaphragm, the fate of the insulin-induced increase of glucose uptake is determined by the activity of the muscle. In resting diaphragm there is primarily an increased incorporation into glycogen (Gemmill, 1940), whereas in contracting diaphragm there is chiefly an increased conversion of glucose to  $\text{CO}_2$  (Fritz, 1960). The increase in glycogen in resting diaphragm is apparently not solely due to the increased glucose uptake (Larner et al., 1959). Although the isolated rat heart was actively contracting, the very consistent rise in cardiac glycogen promoted by insulin in these studies (Table 10), reinforces the suggestion of Larner that insulin especially stimulates glycogen formation in muscle. Even if this were so, a major action of insulin in the isolated perfused rat heart is, undoubtedly, the promotion of glucose transport (Park et al., 1959). This action was not inhibited by an overnight fast of the donor rats (Table 10).

### (c) MANNOSE AND FRUCTOSE

#### Results:

These are shown in Fig. 22. Mannose was handled much the same as glucose although less actively. Fructose uptake and the incorporation of fructose carbon into  $\text{CO}_2$  and glycogen was much less than that of glucose and mannose. Insulin stimulated the uptake of mannose and fructose by about 50%, but its action on fructose- $\text{U-C}^{14}$  was blocked by the presence of equimolar unlabelled glucose (Table 11).



TABLE 11

EFFECT OF GLUCOSE ON INSULIN SENSITIVITY OF FRUCTOSE-u-C<sup>14</sup>

Substrate	Insulin conc.	Fructose uptake	Fructose to	
			glycogen	CO <sub>2</sub>
Fructose-u-C <sup>14</sup> plus glucose both 5 mM/L	0	6.7 $\pm$ 0.7	0.07 $\pm$ 0.01	1.1 $\pm$ 0.03
Fructose-u-C <sup>14</sup> plus glucose both 5 mM/L	2 mu/ml.	6.5 $\pm$ 2.5	0.12 $\pm$ 0.02	0.8 $\pm$ 0.17

Units - uM C6/g.w.w./30 min.

Mean values of 4 hearts  $\pm$  standard error

Rats fed ad. lib.



Discussion:

The mannose handling agrees with findings in adipose tissue (Wood et al., 1959) and suggests that pathways for handling mannose are the same in heart as in other tissues.

Fructose uptake by the isolated perfused rat heart was about one-fifth that of glucose. There is inconstant uptake by the human heart during a fructose infusion (Danforth et al., 1959). These facts make it unlikely that fructose by itself is a fuel for heart muscle contraction, although it can undergo hepatic transformation to triose and pyruvate which in turn are suitable fuels for heart muscle (Hockerts and Lamprecht, 1957). In diaphragm,  $\text{CO}_2$  and glycogen formation are roughly one-quarter as much from fructose as from glucose (Renold and Thorn, 1955). Unless skeletal muscle is considerably more active in its handling of fructose, it is unlikely that fructose could directly restore the upset metabolism of the syndrome of myopathy due to defective muscle glycogenolysis described by McArdle (1951). Its apparent beneficial use in one similar case (Pearson and Rimer, 1959) was probably due to skeletal muscle uptake of products of fructose degradation.

The presence of an equimolar amount of glucose inhibited the action of insulin on fructose uptake and fate (Table II). This agrees with the observations in diaphragm which suggest that fructose uptake occurs chiefly by an insulin-sensitive mechanism which is blocked by the simultaneous presence of glucose (Hackler and Guest, 1953; Leuthardt and Stuhlfauth, 1960).



**(d) SUMMARY**

It was found that:

- (i) Glucose uptake increased with rising perfusate concentrations;
- (ii) Fasting depressed incorporation of label from glucose into  $C^{14}O_2$  and glycogen proportionately more than it diminished glucose uptake;
- (iii) Mannose handling was similar to that of glucose;
- (iv) Fructose uptake was slight;
- (v) Insulin increased hexose uptake by about 50%.



## CHAPTER FIVE

### FREE FATTY ACID METABOLISM

Earlier studies in which arterio-venous differences across dog and human hearts were measured (Gordon et al., 1957; Ballard et al., 1960) suggested the metabolic importance of fatty acids for the heart. The fatty acid components taken up by the heart include: (a) the esterified form measured by triglyceride or chylomicron uptake by the heart (Ballard et al., 1960; Bragdon and Gordon, 1958); and (b) the non-esterified or free fatty acids (F.F.A.). The results presented here concern only the uptake and fate of the highly active (cf. Olson and Vester, 1960) F.F.A. fraction.

In human plasma, approximately two-thirds of the F.F.A. is associated with albumin and up to one-third with the lipoprotein fraction (Olson and Vester, 1960). Albumin was therefore selected as the carrier of the greater amount of F.F.A. The albumin used in this study contained about 800  $\mu\text{M/L}$ . of "endogenous" F.F.A. In a solution of 4 g./100 ml. of albumin. An albumin concentration of 0.5 g./100 ml. (0.5%) was selected so that at perfusate F.F.A. levels usually used (400 - 800  $\mu\text{M/L}$ .), the added or "exogenous" F.F.A. would constitute the major portion of the albumin-bound F.F.A.

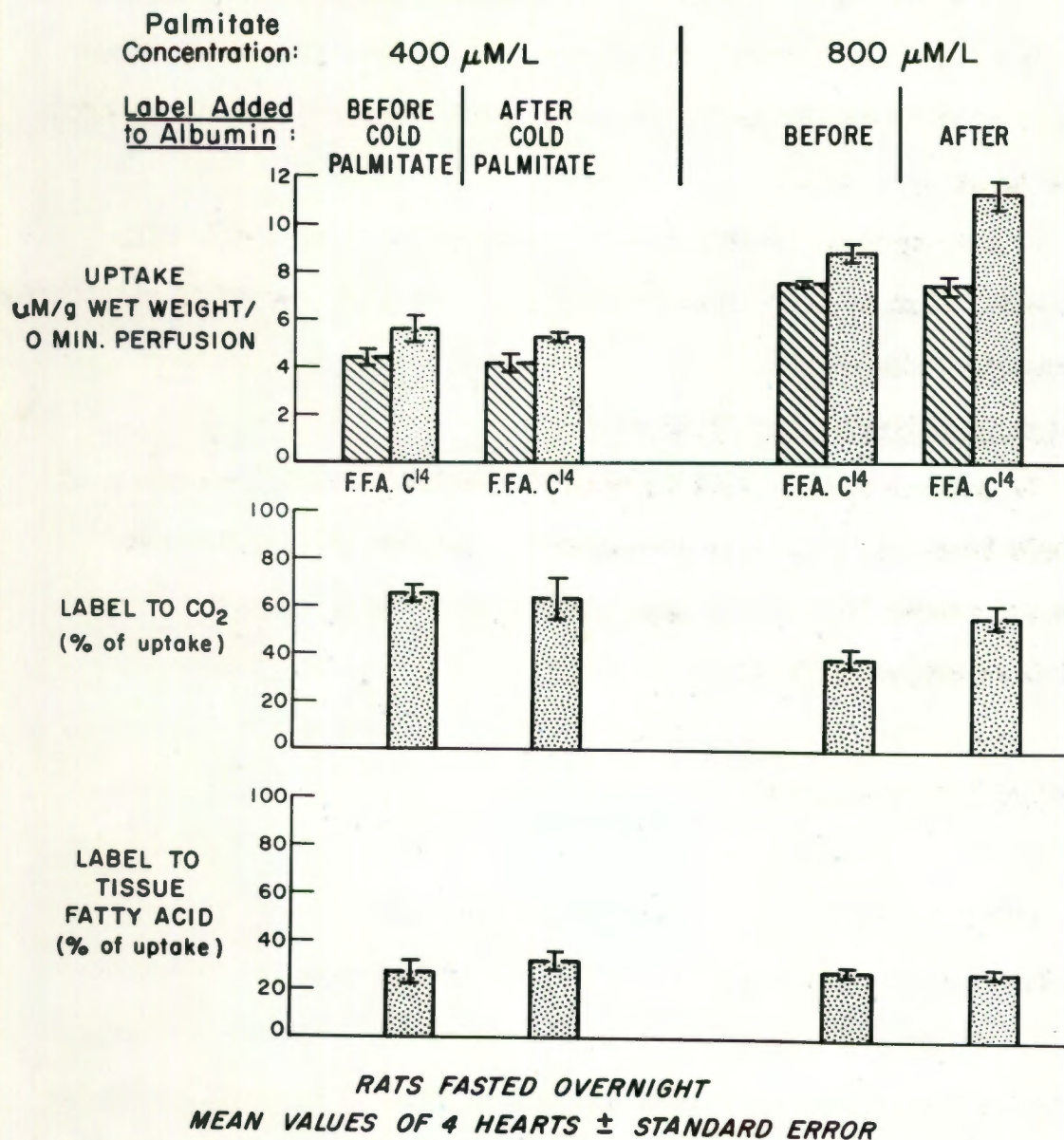
The following investigations were carried out, using hearts taken from rats after an overnight fast (cf. Fig. 16):

#### (1) Cardiac Performance

The performance of the heart was measured by heart rate, force of contraction and coronary flow rate for palmitate concentrations up to 2,500  $\mu\text{M/L}$ . Performance was satisfactory up to 1,500  $\mu\text{M/L}$ .; but at



# **PALMITATE METABOLISM: (1) Method of Adding Label**



**Fig. 23** Method of adding label of more importance at higher palmitate concentration.



2,500  $\mu\text{M/L}$ . It deteriorated during the course of the perfusion. One heart perfused with oleate 64,000  $\mu\text{M/L}$ . (bound to 0.5% albumin) stopped beating after 5 minutes and the coronary flow ceased. Small yellow, lipid-like areas became visible on the hearts surface. Microscopically the arterioles were found to be obstructed.

It was concluded that F.F.A. concentrations above 1,500  $\mu\text{M/L}$ . (bound to 0.5% albumin) were unsatisfactory, and probably caused an obstruction of the coronary vascular bed.

#### (II) Effect of Perfusion Technique on F.F.A.

To exclude the possibility that the perfusion technique affected F.F.A. chain length or degree of unsaturation, samples of albumin-bound palmitate and oleate (800  $\mu\text{M/L}$ .) taken before and after perfusion through the heart were subjected to gas chromatography\*. There were no detectable changes.

#### (III) Method of Adding Label

##### Results:

After making up the 0.5% albumin solution, the label was added to the albumin in amounts less than 10  $\mu\text{M/L}$ ., followed some hours later by 300 or 700  $\mu\text{M/L}$ . of the unlabelled ("cold") palmitate. The final F.F.A. concentration, including the "endogenous" quantity of 100  $\mu\text{M/L}$ ., was therefore just over 400 or 800  $\mu\text{M/L}$ . The above procedure was then reversed and the label added last. The palmitate uptake (Fig. 23) was assessed by: (a) titration and (b) by determining the disappearance of radio-activity from the perfusate during the perfusion, from which the " $^{14}\text{C}$  palmitate uptake" was deduced by assuming that the palmitate specific activity did not change

\* By courtesy of Dr. M. Hegstedt, Harvard School of Public Health.



**TABLE 12**

**F.F.A. BINDING BY ALBUMIN 0.5g/100ml.**

**(based on data by Goodman, 1957 and 1958a)**

<b>Order of binding site</b>	<b>Apparent. association constant *</b>	<b><math>\mu\text{M}</math> FFA bound per <math>\mu\text{M}</math> albumin</b>	<b><math>\mu\text{M}</math> FFA bound/L 0.5% albumin</b>	<b>Total FFA conc. <math>\mu\text{M}</math>/L 0.5% albumin</b>
<b>1st order very tight binding sites</b>	<b><math>6.0 \times 10^7</math></b>	<b>2</b>	<b>145</b>	<b>145</b>
<b>2nd order sites of lesser affinity</b>	<b><math>3.0 \times 10^6</math></b>	<b>5</b>	<b>363</b>	<b>508</b>
<b>3rd order sites of very much less affinity</b>	<b>about <math>1 \times 10^3</math></b>	<b>about 20</b>	<b>about 1450</b>	<b>about 1958</b>

\* calculated for interaction of human serum albumin with palmitate ions at pH 7.45, ionic strength 0.160, 23°C.



during perfusion. For the two methods to give the same result, full equilibration of the labelled and unlabelled palmitate would be necessary.

### Discussion:

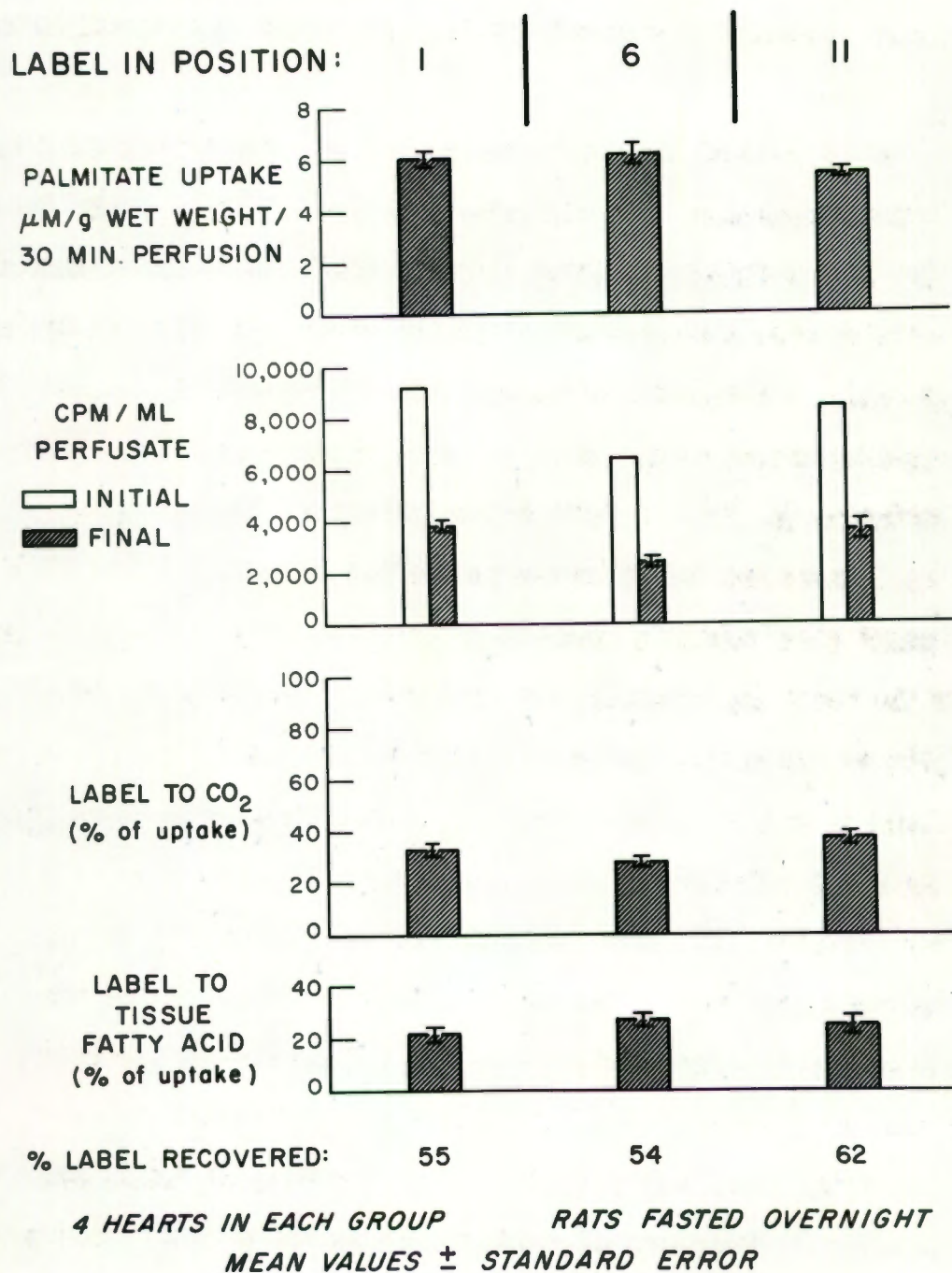
The time of addition of label was important at an F.F.A. concentration of 800  $\mu\text{M/L.}$ , when addition at the end increased label uptake and percentage incorporation into  $^{14}\text{CO}_2$ . These results have been reviewed and extended (Evans et al., 1961) in the light of the description by Goodman (1958 a) of three classes of F.F.A. binding sites on each albumin molecule. The quantity of palmitate that each order could hold in a 0.5% albumin solution is shown in Table 12. In preparing an F.F.A. solution of final concentration 400  $\mu\text{M/L.}$ , the original albumin was not F.F.A.-free but contained endogenous F.F.A., (about 100  $\mu\text{M/L.}$  by titration), which presumably occupied all or most of the very tight binding sites of the first order (calculated to hold 145  $\mu\text{M/L.}$ ). The second class binding sites could cope with enough F.F.A. to make the final concentration over 500  $\mu\text{M/L.}$  Therefore whether the label was added before or after the unlabelled palmitate, it would be bound to sites of the same (second) order. The time of addition was consequently not important.

However, in constituting a F.F.A. solution of final concentration 800  $\mu\text{M/L.}$ , both second and third orders of binding sites were involved (Table 12). If the label was added after unlabelled palmitate had fully "occupied" the second order of sites, it could be bound only by third order sites. This might have accounted for the greater uptake and percentage incorporation into  $^{14}\text{CO}_2$  than obtained when the label was added first and bound with greater affinity by second order sites.

At concentrations of F.F.A. which would exceed the total albumin binding capacity (2,400 and 64,000  $\mu\text{M/L.}$ ) the performance of the isolated



**PALMITATE METABOLISM: (2) Myocardial Handling  
of Palmitate 1, 6, and 11 - C<sup>14</sup> (800  $\mu$ M/L)**



**Fig. 24** Similar metabolism of palmitate labelled in various positions.



rat heart was poor, possibly because the F.F.A., not in solution, was free to form macromolecular aggregates (Boelj, 1949) which obstructed coronary flow.

The uptake of the label was greater than the titratable F.F.A. uptake. This suggested: (a) titration error, which was excluded by repetition with a different method (Gordon, 1957); or (b) breakdown of cardiac lipids with diffusion of unlabelled fatty acid into the perfusate, thus decreasing the titratable uptake; this was regarded as unlikely because chromatography showed no change in the type of perfusate F.F.A. before and after perfusion (p. 30). A further possibility was that labelled molecules were handled more actively than the unlabelled ones which included the "endogenous" group probably bound to first order sites. It may be questioned whether the heart can actually take up the most tenaciously-bound F.F.A., especially if the cardiac cellular mechanisms for F.F.A. uptake resemble those found in red cells which cannot compete with albumin for F.F.A. until the first order of binding sites is saturated (Goodman, 1958 a). In diaphragm (Fritz et al., 1958) oxidation of palmitate-1- $C^{14}$  is depressed when albumin is added in excess of 1  $\mu$ M. albumin to 7  $\mu$ M. palmitate. In isolated rat heart mitochondria, palmitate uptake is much reduced by higher albumin concentrations (Fritz and Kaplan, 1960 a).

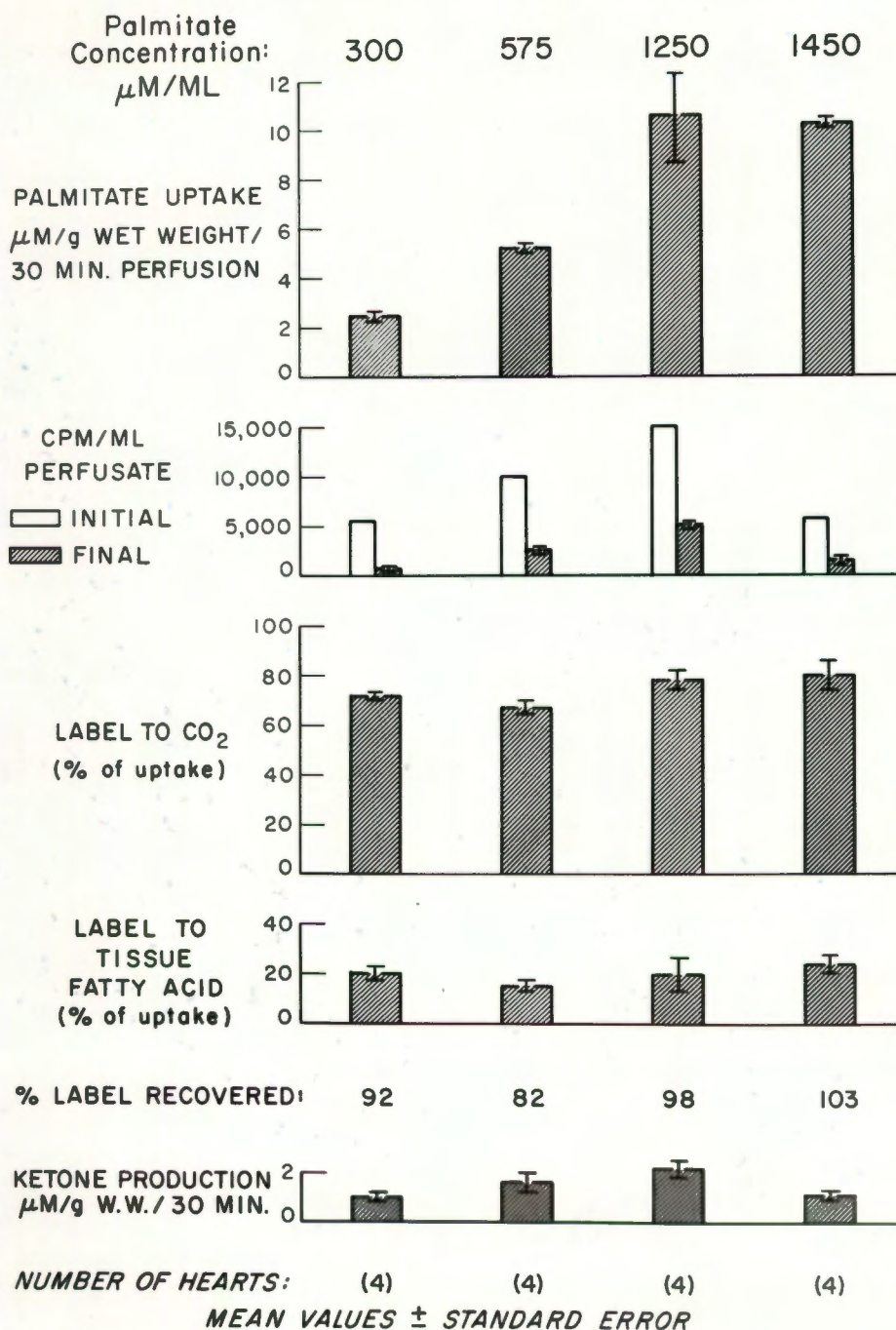
It was concluded that the cardiac handling of F.F.A. was influenced by the tightness of the binding with which it was held to the albumin.

In all further experiments reported here, with the exception of the experiment concerning oleic acid, the label was added to the albumin after the unlabelled "cold" F.F.A. This allowed study of F.F.A. metabolism



# **PALMITATE METABOLISM: (3) Effect of Palmitate - 1 - C<sup>14</sup> Concentration**

*LABEL ADDED TO ALBUMIN 0.5 g/100ML AFTER COLD PALMITATE*



**Fig. 25** Effect of increasing palmitate concentration.



under conditions favouring maximum uptake of label and incorporation into  $^{14}\text{CO}_2$ . However, it must be stressed that the evidence is that such label was not fully equilibrated with all F.F.A. present on the albumin.

(iv) Comparative Metabolism of Palmitate 1-, 6-, and 11- $\text{C}^{14}$

As shown in Fig. 24, the myocardial handling of the label was the same irrespective of the position of the label. It was concluded that terminal decarboxylation of palmitate did not occur during the conditions of the perfusate, and that the fate of the carboxyl label was a reasonably accurate index of the whole palmitate chain.

(v) Effect of Perfusate Palmitate Concentration

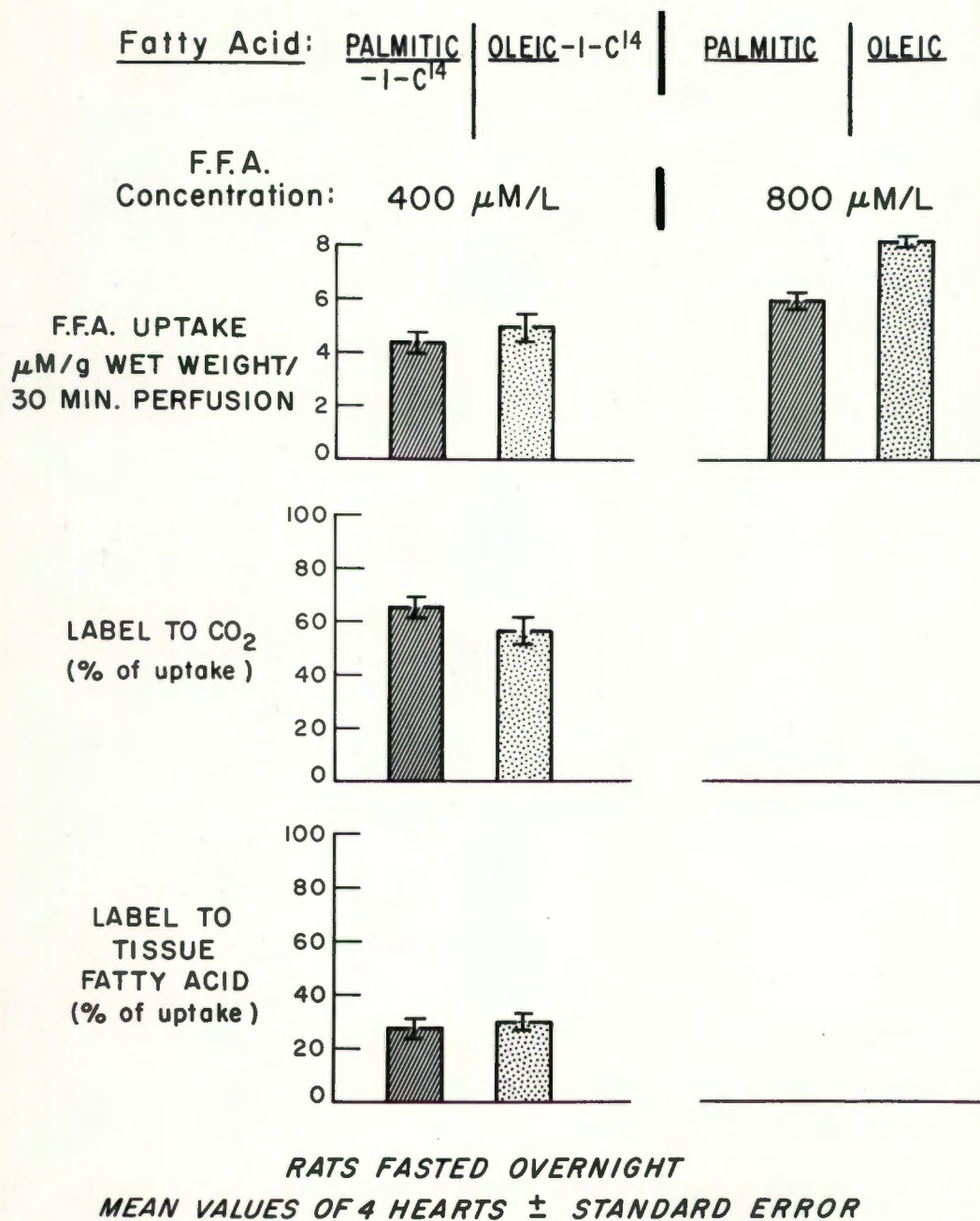
Increasing concentrations of palmitate (Fig. 25) resulted in an increasing titratable uptake up to 1,250  $\mu\text{M/L}$ . A constant proportion of label, between 65 and 80%, was incorporated into  $^{14}\text{CO}_2$  while 15 - 24% was recovered in the tissue fatty acid component. Ketone formation was slight. No incorporation of palmitate label into glycogen was detected.

The approximately linear increase of palmitate uptake up to 1,250  $\mu\text{M/L}$ . appears to be contrary to the suggestion that at higher concentrations the larger part of the palmitate was loosely bound and that this facilitated capture of the palmitate by the heart. On the other hand, with an increasing palmitate concentration the mechanisms for its uptake must have become increasingly saturated until there was no increase in uptake at concentrations above 1,250  $\mu\text{M/L}$ . It could be argued that this tendency to decreased uptake was balanced out by the greater ease of uptake of the greater amount of loosely bound palmitate at higher concentrations; thus two opposing factors could cancel each other to produce a linear uptake.

The large fraction of label incorporated into  $\text{C}^{14}\text{O}_2$  was



# METABOLISM OF OLEIC ACID: (1) Comparison with Palmitic Acid



**Fig. 26** Oleic acid uptake slightly greater than that of palmitate.



cautiously interpreted since the method of preparing labelled albumin-bound palmitate enhanced incorporation of label into  $C^{14}O_2$ . To fully establish the relationship between titratable uptake and  $C^{14}O_2$  formation under more "physiological" conditions would require extended studies: using: (a) albumin liberated of "endogenous" F.F.A. by extraction with acetic acid in iso-octane (Goodman, 1957); (b) higher albumin concentrations; and (c) simultaneous addition of labelled and unlabelled F.F.A to the albumin. Using these conditions it might be possible to confirm Lehninger's observation, in 1945, that higher fatty acid concentrations (above 1,000  $\mu M/L$  of octanoate) inhibited fatty acid oxidation.

The small quantity of ketone formed supports the finding in heart homogenates that fatty acid oxidation goes to completion and ketone formation is negligible (Lehninger, 1946; Fritz, 1961).

#### (vi) Oleic Acid Metabolism

Oleic acid was studied as an example of an unsaturated long-chain fatty acid (Fig 26). The oleate uptake was <sup>slightly</sup> greater than that of palmitate, comparable to findings in vivo (Bing, 1960), but the overall fates of oleate and palmitate were similar.

#### (vii) Conclusions

It was concluded that:

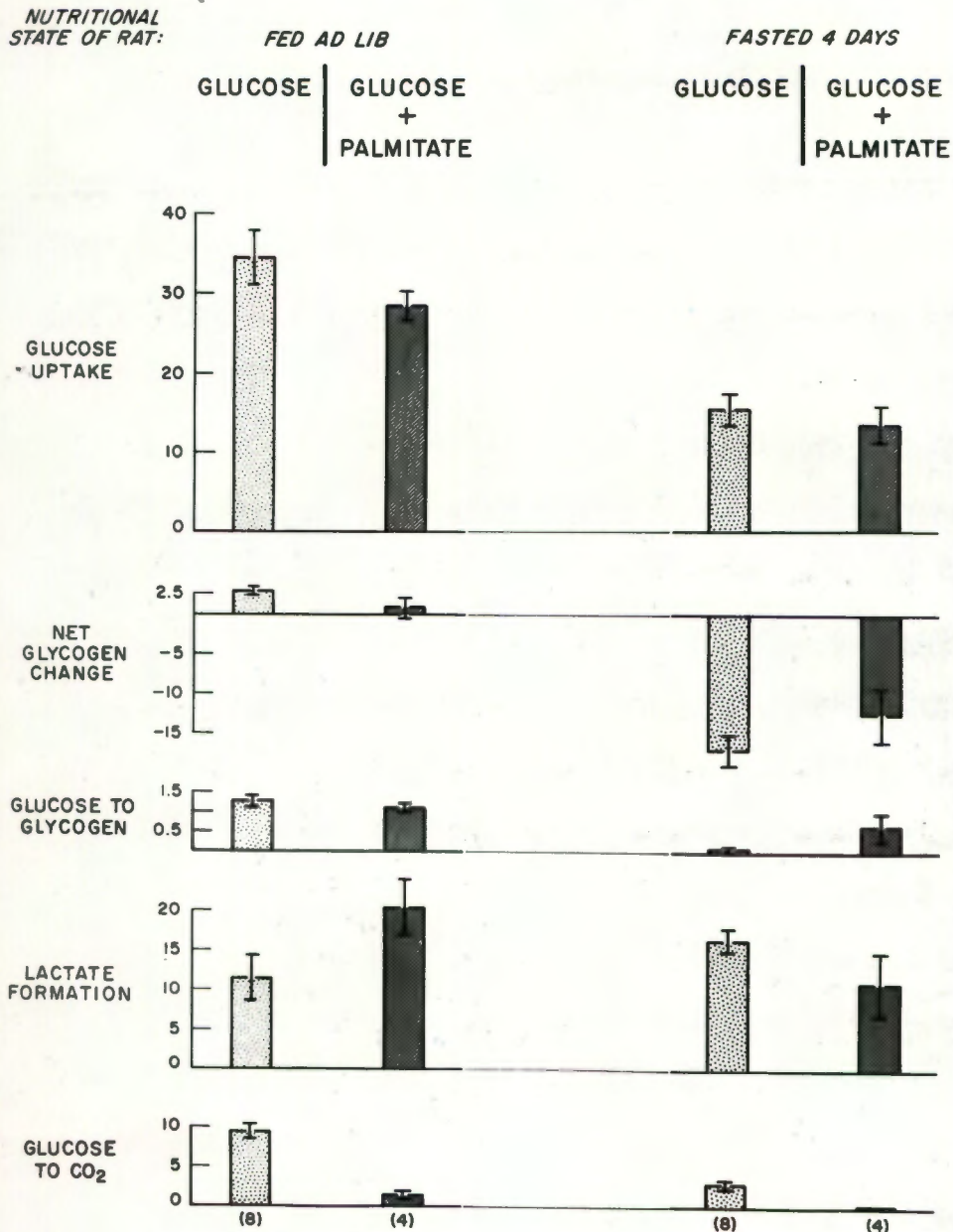
- (i) Perfusion with palmitate bound to 0.5% albumin was compatible with good function of the isolated rat heart up to a concentration of 1,500  $\mu M/L$ ;
- (ii) The perfusion technique did not induce changes in palmitate or oleate which could be detected by gas chromatography;



- (iii) Label in the carboxyl position was an adequate index of the overall metabolism of the whole palmitate chain;
- (iv) The relative times of addition of the labelled and unlabelled palmitate to 0.5% albumin influenced the subsequent uptake and fate of the label during the perfusion period;
- (v) Maximum palmitate uptake was reached at a concentration of about 1,250  $\mu\text{M/L.}$ ; and with palmitate-1- $\text{C}^{14}$  prepared in a defined fashion, 65 - 80% of the label was incorporated into  $\text{C}^{14}\text{O}_2$ , while the rest was recovered in tissue fatty acids.
- (vi) Oleate uptake was slightly greater than that of palmitate, but the metabolic fates were similar.



**GLUCOSE METABOLISM: (6) Effect of Palmitate 750  $\mu$ M/L on  
Glucose - u - C<sup>14</sup> 5MM/L.**



UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 min. PERFUSION  
MEAN VALUES  $\pm$  STANDARD ERROR ( ) = NUMBER OF HEARTS

**Fig. 27** Albumin-bound palmitate reduced glucose oxidation and increased lactate production (fed state) and incorporation of glucose into glycogen (fasted state).



## CHAPTER SIX

INTERACTION OF GLUCOSE AND  
PALMITATE METABOLISM

As carbohydrate and fatty acids appear to be the chief cardiac fuel taken up by the heart (Bing et al., 1958), the simultaneous handling of glucose and palmitate was studied to trace the fate of each in the presence of the other.

(1) Addition of Palmitate to Glucose-u-C<sup>14</sup>

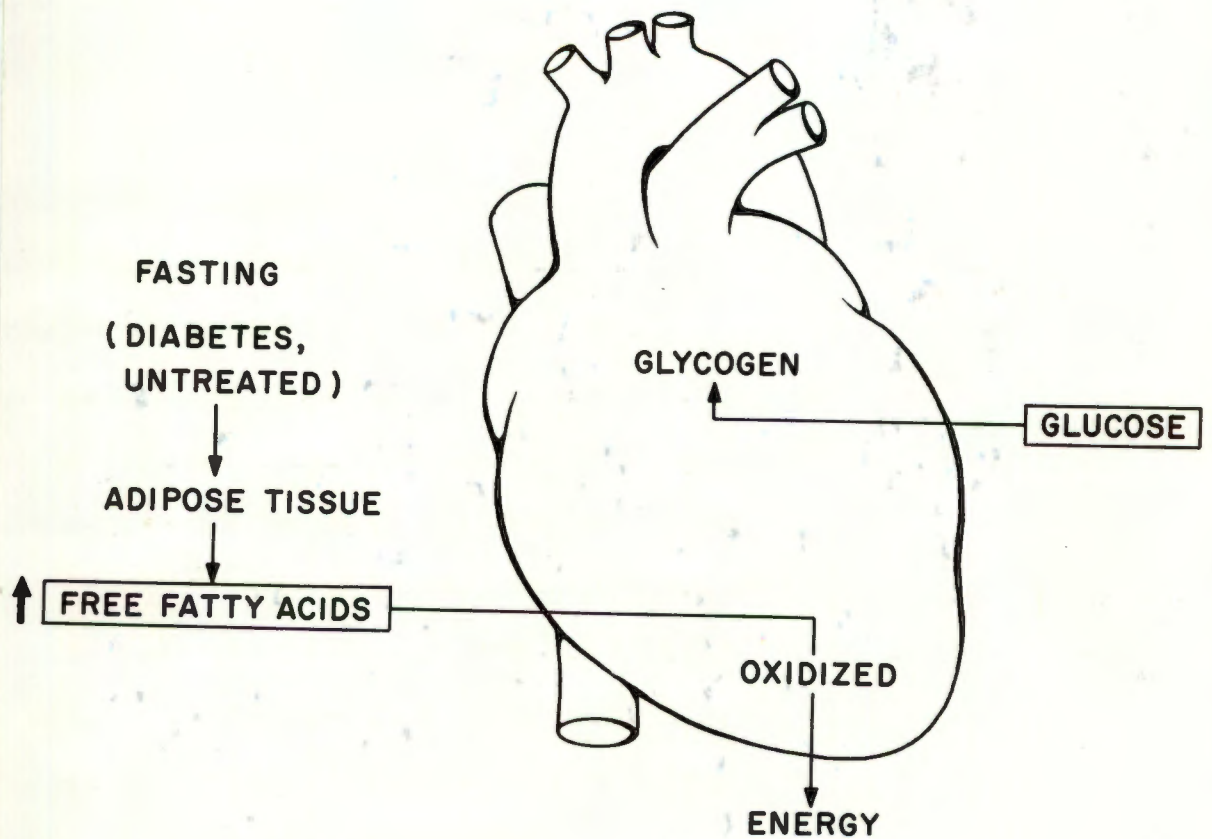
The effect of palmitate (750  $\mu$ M/L. bound to 0.5% albumin) on glucose-u-C<sup>14</sup> (5 mM/L.) metabolism by hearts from rats fed ad lib. and fasted four days, was studied (Fig. 27). In these conditions, palmitate grossly reduced glucose oxidation from  $9.51 \pm 0.85$  to  $1.56 \pm 0.29$  in the fed state, and from  $2.73 \pm 0.45$  to  $0.23 \pm 0.05$  in the fasted state (units =  $\mu$ M. C6/g.w.w./30 min.). Simultaneously lactate formation was increased in the fed state and incorporation of C<sup>14</sup> label from glucose into glycogen increased in the fasted state. The addition of palmitate increased pyruvate production from  $0.7 \pm 0.08$  to  $1.0 \pm 0.10$   $\mu$ M. C6/g.w.w./30 min. in the fed state. These concomitant changes in glucose fate argue against the palmitate effect on glucose oxidation merely being due to dilution of labelled CO<sub>2</sub> precursors.

In the fasted state palmitate promoted incorporation of glucose label into glycogen and decreased glycogen breakdown. This tendency to stop glycogen break-down in perfused hearts from fasted rats therefore reverts the situation towards that in vivo in which cardiac glycogen increases in fasting and diabetes (Cambridge, 1913; Evans, 1933). If F.F.A. also inhibits glucose oxidation in vivo and promotes glycogen synthesis, this could explain the rise of cardiac glycogen with fasting.

A simplified and schematic version of this proposed



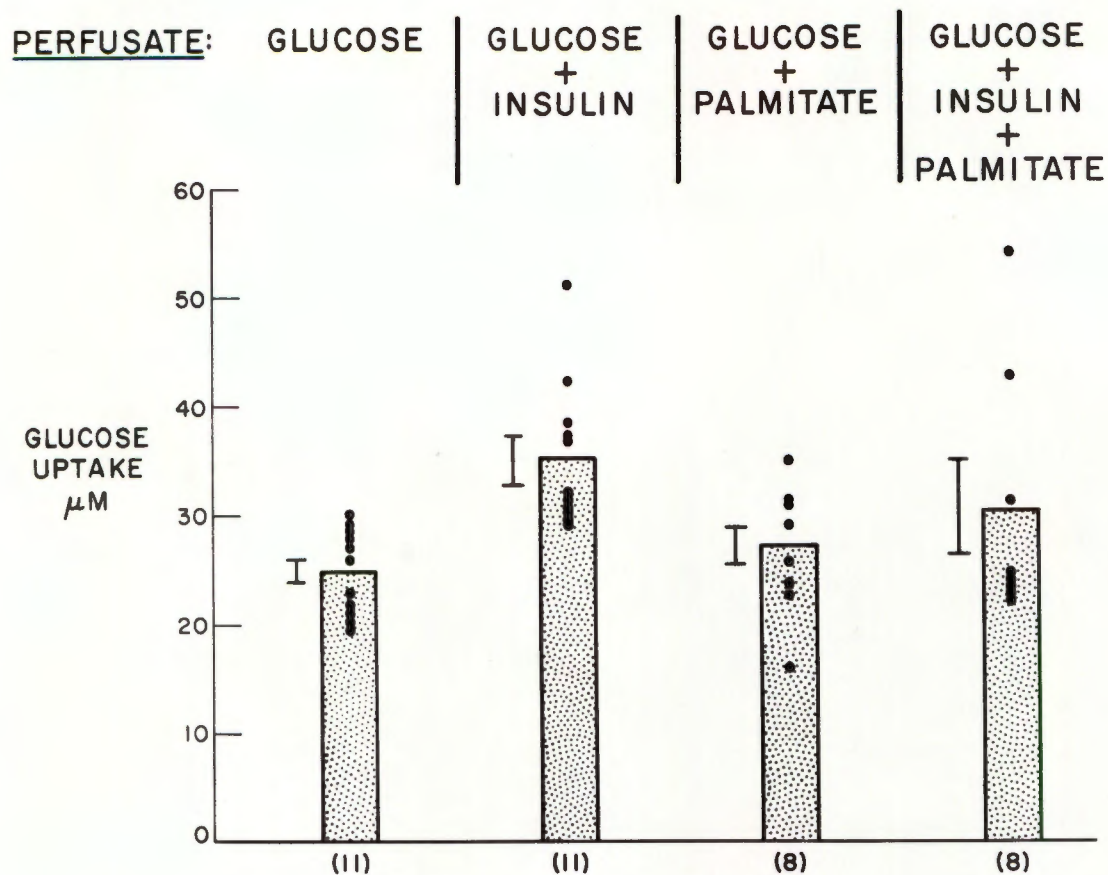
## MYOCARDIAL METABOLISM: POSSIBLE EFFECT OF FASTING



**Fig. 26** Possible cause of increased cardiac glycogen in fasting and untreated diabetes.



**GLUCOSE METABOLISM: (7) Relation of Palmitate  
to Effect of Insulin on Glucose Uptake**



GLUCOSE 5 mM/L.

PALMITATE 800  $\mu\text{M}$ /L (Albumin 0.5 g/100 ml)

INSULIN  $2 \times 10^{-3}$  units/ml

ANIMALS FED AD LIB

UNITS =  $\mu\text{M}$  GLUCOSE EQUIVALENT/g WET WEIGHT/30 min PERFUSION

MEAN VALUES  $\pm$  STANDARD ERROR ( ) = NUMBER OF HEARTS

**Fig. 29** Effect of palmitate on insulin-sensitivity of glucose uptake.



theory is shown in Fig. 28. This suggestion is supported by Bowman (1959) who found that oleic acid feeds elevated cardiac glycogen in fasting, hypophysectomized rats who otherwise lost it. Lackey and co-workers (1947) could increase the cardiac glycogen by intravenous injection of ketone bodies. This finding suggested to Olson and Schwartz (1951) that cardiac carbohydrate was spared by preferential utilization of ketone bodies.

### (II) Palmitate Effect in Fed State

The above unequivocal results were obtained with glucose and palmitate concentrations corresponding to those found in rats fasted for four days rather than in non-fasted rats (Fig. 14). To mimic the fed state, the effect of palmitate 300  $\mu$ M/L. on glucose 7.5 mM/L., in presence of insulin 2  $\mu$ u/ml., was studied. (Table 13).

#### Results and Discussion:

The addition of palmitate reduced glucose label incorporation into  $C^{14}O_2$  from  $12.5 \pm 1.4$  to  $5.3 \pm 1.1$   $\mu$ M. C6/g.w.w./30 min. The F.F.A. still clearly reduced glucose oxidation, but dilution could not be excluded as a cause of this decrease because of the absence of definite changes in lactate or glycogen formation. The F.F.A. uptake of 3  $\mu$ M/g.w.w./30 min. in the fed state and in the presence of glucose 7.5 mM/L., was similar to that obtained at the same F.F.A. concentration (300  $\mu$ M/L.) in the absence of glucose and in the fasted state. (Fig. 25).

### (III) Effect of Palmitate on Glucose Uptake

#### Results and Discussion:

To clarify any effect of palmitate on glucose uptake, the uptake was studied in the presence of insulin, palmitate and palmitate plus insulin (Fig. 29). Palmitate did not alter glucose uptake, but reduced the effect



**TABLE 13****EFFECT OF PALMITATE (300  $\mu$ M/L) ON GLUCOSE-u- $C^{14}$  7.5 mM/L****IN THE PRESENCE OF INSULIN (2 mU/ml.)****RATS FED AD. LIB.**

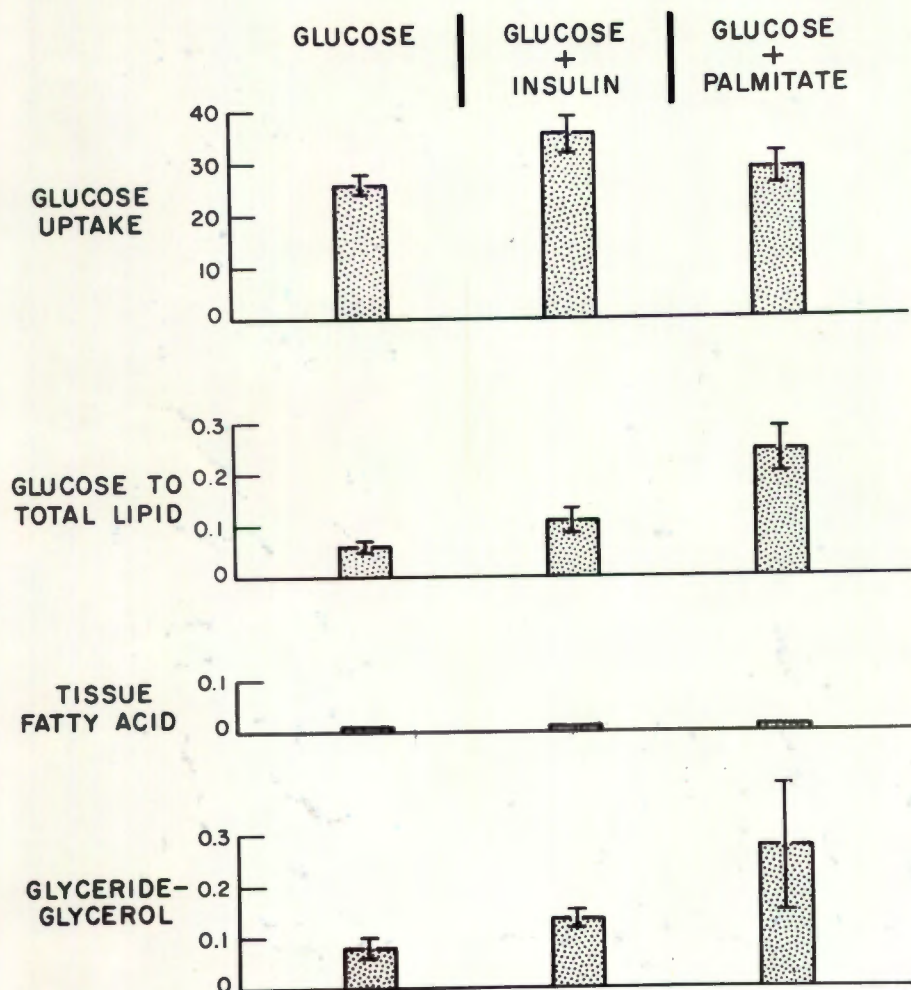
	Glucose	Glucose + Palmitate
Glucose uptake	54.0 $\pm$ 3.5	50.7 $\pm$ 7.3
Net glycogen change	4.6 $\pm$ 0.8	6.0 $\pm$ 1.5
Glucose to glycogen	5.0 $\pm$ 1.9	5.5 $\pm$ 0.5
Lactate production	18.9 $\pm$ 2.4	22.9 $\pm$ 5.8
Glucose to CO <sub>2</sub>	12.5 $\pm$ 1.4	5.5 $\pm$ 0.5
Recovery of carbohydrate	67%	67%
F. P. A. uptake uM. C16/g.w.w./30 min.		3.0 $\pm$ 1.4

Units = uM.C6/g.w.w./30 min.

Mean values of 8 hearts  $\pm$  standard error.Glucose to CO<sub>2</sub> = incorporation of glucose carbon into CO<sub>2</sub>; measured  
by incorporation of label from glucose-u- $C^{14}$  into  
 $C^{14}O_2$ .



**GLUCOSE METABOLISM: (8) Lipid Formation from**  
**Glucose-u-C<sup>14</sup> 5MM/L.**



*RATS FED AD LIB      INSULIN =  $2 \times 10^{-3}$  u/ML*  
*PALMITATE = 850  $\mu$ M/L, ALBUMIN 0.5 g/100 ML*  
*UNITS =  $\mu$ M GLUCOSE EQUIVALENT/g WET WEIGHT/30 MIN.*  
*MEAN VALUES OF 4 HEARTS  $\pm$  STANDARD ERROR*

**Fig. 30** Glyceride-glycerol formation from glucose accounts for most of glucose label recovered in lipid.



of insulin on glucose uptake. This relative insensitivity to insulin was possibly consequent on an accumulation of glycolytic intermediaries which could not be oxidized because of the presence of the palmitate.

(iv) Effect of Palmitate on Lipid Formation from Glucose- $u\text{-C}^{14}$

Results:

Incorporation of glucose carbon into lipid was studied in the presence of insulin and palmitate (Fig. 30). Insulin increased glucose uptake and incorporation into lipid proportionately. The addition of palmitate to glucose did not influence glucose uptake, but did significantly increase its incorporation into lipid. Further analysis of the radio-active lipid showed that the non-saponifiable fraction contained none of the radio-activity, while the tissue fatty acid component contained very little. Therefore it was no surprise to find that there was measurable incorporation of glucose label into the glyceride-glycerol fraction and that this incorporation was increased by palmitate.

Discussion:

Lipid formation from glucose, although qualitatively of great interest, is quantitatively minimal in this tissue (less than 1% of the glucose uptake). This agrees with the minimal incorporation of acetate into fat in rat heart slices (Pearson et al., 1949).

Virtually none of the lipid synthesis could be accounted for by fatty acid synthesis, which is usually, but not invariably, associated with rapid T.P.N.H. generation by the hexose monophosphate shunt (Fritz, 1961). In rat heart muscle, glucose-6-phosphate dehydrogenase activity is low (Glock and McLean, 1954), presumably indicating a poorly developed shunt, compatible with minimal cardiac lipogenesis.



**PALMITATE METABOLISM: (4) Effect of Glucose 5MM on  
PALMITATE-1-C<sup>14</sup> 700  $\mu$ M/L**

**NUTRITIONAL  
STATE OF RAT:**

**FED AD LIB**

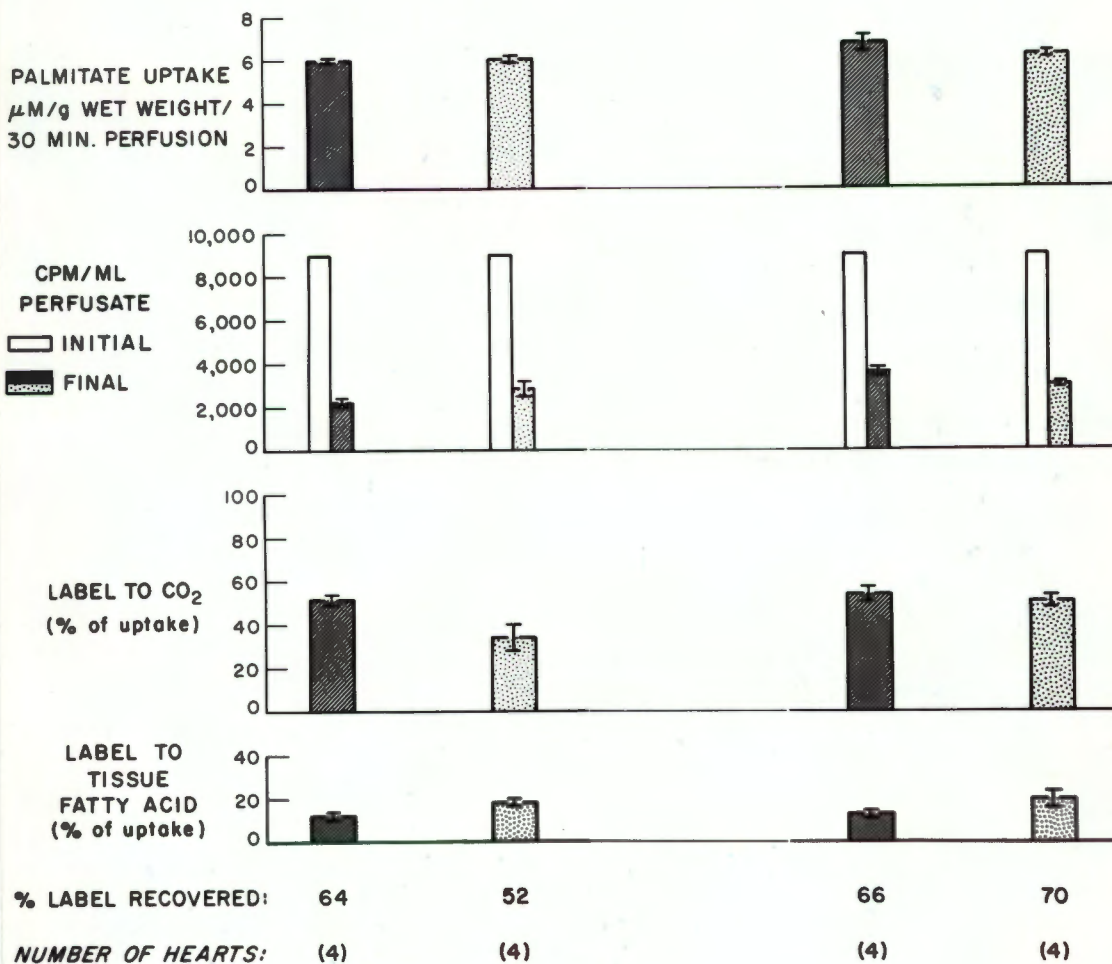
**FASTED 4 DAYS**

**PALMITATE**

**PALMITATE  
+  
GLUCOSE**

**PALMITATE**

**PALMITATE  
+  
GLUCOSE**



MEAN VALUES  $\pm$  STANDARD ERROR

Glucose concentration = 5 mM/L.

**Fig. 31** No effect of glucose on palmitate oxidation in fasted state; in fed state slight decrease of oxidation. In both states slight increase in palmitate incorporation into tissue fatty acid.



(v) Effect of Glucose on Palmitate-1-C<sup>14</sup>

Results:

The effects of glucose (5 mM/L.) on palmitate-1-C<sup>14</sup> (700 uM/L., bound to 0.5% albumin) are shown in Fig. 31. There was: (a) no change in titratable F.F.A. uptake; (b) a decreased incorporation of palmitate label into CO<sub>2</sub> ( $51.7 \pm 2.5$  vs.  $34.0 \pm 6.4\%$ ) in the fed state, but no such effect in the fasted state; and (c) a slight but consistent rise in the recovery of label in tissue fatty acid.

Discussion:

The lack of effect of glucose on palmitate uptake apparently contradicts the observation of Gordon and Cherkas (1956) that a glucose infusion inhibited F.F.A. uptake by the human heart. However, in their single observation, the arterial F.F.A. level dropped from 960 to 310 uM/L. during the glucose infusion. The isolated heart has the advantage of perfect control of the initial substrate levels.

Glucose increased the incorporation of palmitate label into tissue fatty acid; this is compatible with the increased glyceride-glycerol formation from glucose- $u\text{-C}^{14}$  in the presence of palmitate, and suggests that at least some of the tissue fatty acid formed during the perfusion was glyceride.

(vi) Glucose - F.F.A. Interrelations in Other Systems

The present finding that palmitate inhibits glucose oxidation should be compared and, if possible, reconciled with the data obtained by other workers, demonstrating that in dog and man carbohydrate makes the major contribution to myocardial oxidation in the fed state (cf. Fig. 32). In the presence of glucose 5 mM/L. and palmitate 700 uM/L., about one



Effect of Feeding and Fasting Upon  
Myocardial Substrate Utilization  
In Dog and Man (Olson, A. N.Y. Acad 72:471, 1958)

Condition	Fed	Fasted
A-V Difference		
O <sub>2</sub> ml. per cent	10.80	11.50
CHO MM C-3/L	1.49	0.51
FFA MM C-16/L	0.01	0.12
% of O <sub>2</sub> uptake accounted for by		
CHO	92	30
FFA	5	58
Myocardiac Respiratory Quotient Approaches	1.0	0.7

**Fig. 32** In vivo the heart utilizes carbohydrate in fed state, but F.F.A. in fasted state.



one-third of the palmitate label is oxidized (Fig. 31). Assuming that this figure was not exceeded at concentrations corresponding to those found in rats fed ad lib. (glucose 7.5 mM/L. and palmitate 300 uM/L.) then one-third of the palmitate uptake (cf. Table 13) was oxidized, i.e.,  $(3 \times 1/3 \times 16)$  or 16 carbon units/g.w.w./30 min. were oxidized according to the reaction:



At the same time 5.5 uM. C<sub>6</sub>/g.w.w./30 min. of glucose were oxidized, i.e.,  $(5.5 \times 6)$  or 33 carbon units/g.w.w./30 min. of glucose were oxidized according to the equation:



Therefore 33 O<sub>2</sub> units were used for 33 glucose carbon units.

Therefore  $\frac{33}{33 + 23} \times 100\%$  or 58% of the measured oxidation was supplied by glucose. Thus it is possible for palmitate to reduce glucose oxidation by half (Table 13) and yet for glucose to be the major substrate for oxidation. In other words, the very slight F.F.A. uptake noted by Olson in the fed state (Fig. 32) could still inhibit glucose oxidation without diminishing the role of carbohydrate as chief cardiac fuel in these conditions. Such a postulated inhibition of glucose oxidation could perhaps be demonstrated by artificially raising the circulating F.F.A. levels from the low values associated with the fed state.

Further agreement with *in vivo* data is possible by invoking Goodman's concept of albumin binding sites of various orders (Table 12). At a F.F.A. concentration of 300 uM/L., bound to 0.5% albumin, about half the F.F.A. is held to first and half to second order sites. If, however, the albumin were in a concentration greater than 1 g/100 ml.



(as it would be in the blood used for the data in Fig. 14) then, at 300  $\mu\text{M/L.}$ , all the F.F.A. would be tightly bound. This might considerably reduce F.F.A. uptake. In the data obtained by Gordon and Cherkas (1956) on the subject already referred to, there was indeed no measurable uptake at an F.F.A. level of 310  $\mu\text{M/L.}$

Williamson (1960) studied the competition between aceto-acetate (5 and 10  $\text{mM/L.}$ ) and glucose (8.35  $\text{mM/L.}$ ) in isolated perfused hearts taken from rats of unspecified nutritional state. The glucose uptake, allowing for net glycogen formation but not for other non-oxidative fates, accounted for only 32% the oxygen consumption as measured by a platinum electrode. On the other hand, the acetate uptake (correcting for  $\beta$ -hydroxybutyrate formation) could account for 62% of the oxygen uptake. He concluded that aceto-acetate competed favourably with glucose oxidation.

In heart homogenates prepared from rats fed ad lib., Allen and co-workers (1955) found the glucose 5  $\text{mM/L.}$  failed to inhibit and possibly stimulated the oxidation of carboxyl-labelled palmitate 50 - 250  $\mu\text{M/L.}$  The findings were similar when endogenous cardiac lipid was labelled by palmitate.

In surviving diaphragm, Fritz and Kaplan (1960) had different results from those presented here. They worked with lighter rats (150-200 g.) and used 0.1% albumin to bind palmitate 97-1,000  $\mu\text{M/L.}$  The incorporation of palmitate label into  $\text{C}^{14}\text{O}_2$  was increased by fasting donor rats for three or more days, in contrast to the lack of effect of a similar fast on palmitate handling by the isolated rat heart (Fig. 14). They also found that the increase in palmitate oxidation after fasting



was minimized by the simultaneous presence of glucose 5 mM/L., while in the fed state glucose had no effect on palmitate oxidation.

Neptune and his co-workers (1959) studied the competition between butyrate (4,450 uM/L.) and glucose (8.4 mM/L.) in the diaphragm. In the fed state, butyrate contributed 54% of the  $\text{CO}_2$  production and glucose 29%, while in the fasted state butyrate contributed 67% and glucose only 12%. These fatty acid levels are, however, much higher than those used in this study.

In skeletal muscle, Fritz and his associates (1958) found that glucose 5 and 10 mM/L. did not decrease the incorporation of palmitate-1- $\text{C}^{14}$  into  $\text{C}^{14}\text{O}_2$ . These experiments are of especial importance because they were performed on tissue taken from fed rats, and the initial palmitate concentration in the medium was 95 uM/L. The albumin to palmitate ratio was 1:10, indicating that they were working with palmitate bound at least in part to third order sites (cf. Table 12) which makes their albumin to palmitate ratio similar to that used in Figs. 29 and 30 (at 750 uM/L. in 0.5% albumin, the albumin to palmitate ratio is 1:10.4).

The studies of these workers support the work of Andres et al., (1956) which stresses the importance of non-carbohydrate material as substrate for oxidation in peripheral muscle. The liberal lactate formation from glucose (about one-third of the glucose uptake) found by these workers is compatible with F.F.A. diverting glucose from oxidation to lactate formation.

Comparative studies in insects and birds (Drummond and Black, 1960) also stress the role of lipid as fuel. Fritz, in his recent review (1961), concludes that the traditional concept of carbohydrate as the



predominant fuel for working muscle is challenged.

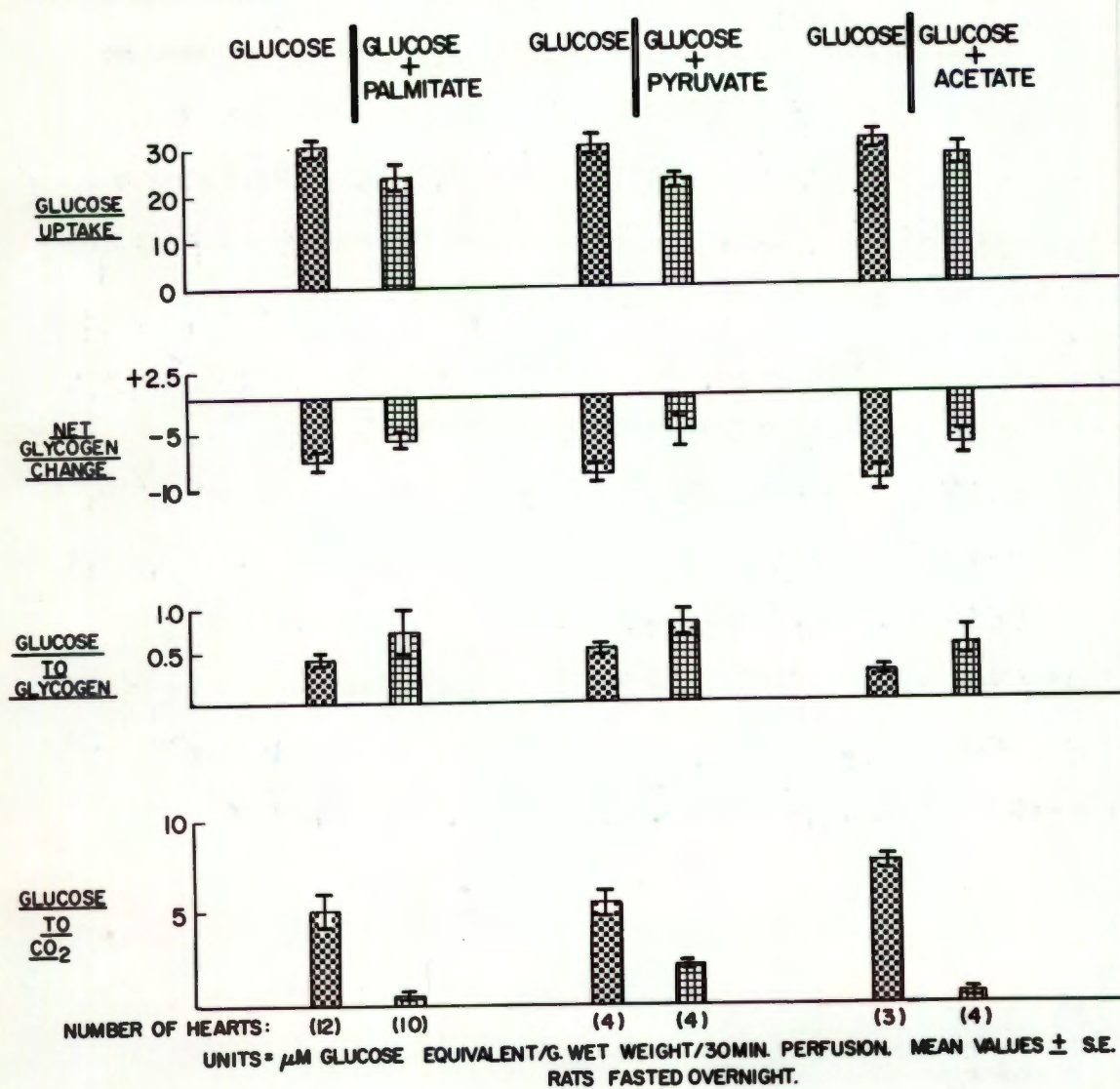
(viii) Summary:

It is concluded that:

- (i) Palmitate (750  $\mu\text{M/L}$ ., bound to 0.5% albumin) was a major controlling factor in the handling of glucose 5  $\text{mM/L}$ . by the heart in the conditions of this work; its major effect was inhibition of glucose oxidation;
- (ii) In conditions favouring optimum carbohydrate usage (the fed state, the presence of insulin, a high perfusate glucose concentration and a low palmitate concentration), long-chain fatty acid still reduced glucose oxidation;
- (iii) The effect of palmitate on glucose uptake was variable; palmitate appeared to diminish the response of the uptake to insulin;
- (iv) Palmitate enhanced lipid and glyceride-glycerol formation from glucose;
- (v) The addition of glucose did not reduce palmitate oxidation in the fasted state, but in the fed state it reduced palmitate oxidation by one-third;
- (vi) These findings can be partially reconciled with current knowledge of the major cardiac fuels in dog and man, and are compatible with reported findings in skeletal muscle.



**GLUCOSE METABOLISM (9):** Effect of Palmitate 400  $\mu$ Eq/L, Pyruvate 5mM, and Acetate 5mM on Glucose -U- $C^{14}$  5mM.



Pyruvate, acetate, glucose = 5mM/L.

**Fig. 33** Palmitate, pyruvate and acetate all reduce glucose oxidation (rats fasted overnight).



CHAPTER SEVENMECHANISM OF GLUCOSE - PALMITATE INTERACTION

The failure of added glucose to inhibit palmitate oxidation in hearts from fasted rats, as reported in Chapter 6, could be criticized on the following grounds: (a) the disparity between the handling of the labelled and the unlabelled palmitate (cf. Fig. 23) was greater at the concentration used (800  $\mu\text{M/L.}$ ) than it would have been at lower concentrations such as 400  $\mu\text{M/L.}$ ; (b) the glucose uptake was unduly depressed by the very long fast of four days; (c) the effect of higher glucose concentrations on palmitate metabolism was not studied.

The different design of some of the experiments reported in this chapter counters these objections. The palmitate concentration used was 400  $\mu\text{M/L.}$ , within the range of albumin binding in which the method of addition of label made no difference to the subsequent fate of the label. Furthermore, the rats were fasted overnight only and glucose concentrations of both 5 and 10  $\text{mM.}$  were used, thereby ensuring appreciable glucose uptake.

In addition, further studies were undertaken to delineate the exact metabolic steps involved in the glucose-palmitate interaction.

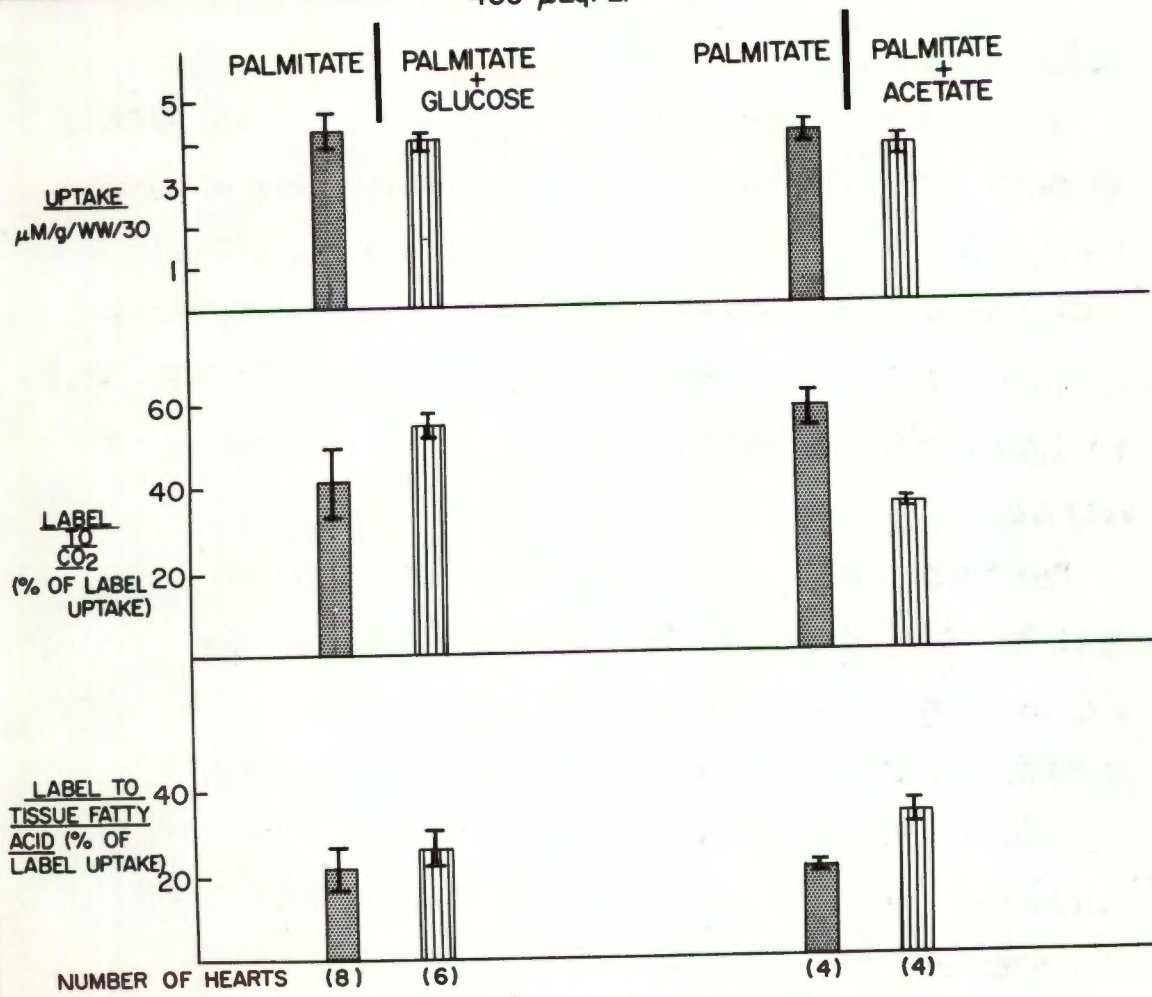
(i) The Effect of Palmitate, Pyruvate and Acetate on Glucose- $\text{u-C}^{14}$

Results:

The metabolism of glucose- $\text{u-C}^{14}$  5  $\text{mM/L.}$  was studied in the presence of palmitate 400  $\mu\text{M/L.}$ , pyruvate 5  $\text{mM/L.}$  and acetate 5  $\text{mM/L.}$  (Fig. 33). These all reduced incorporation of label from glucose into  $\text{C}^{14}\text{O}_2$ , and tended to increase incorporation into glycogen, decrease glycogen breakdown, and reduce glucose



**PALMITATE METABOLISM (5):** Effect of Glucose 5mM and Acetate 5mM on Palmitate 400  $\mu$ Eq/L.



RATS FASTED OVERNIGHT. MEAN VALUES  $\pm$  STANDARD ERROR

Glucose, acetate = 5 mM/L.

**Fig. 34** Effects of glucose and acetate on CO<sub>2</sub> formation from palmitate. Rats fasted overnight.



uptake. Lactate measurements were not available. The most marked inhibition of glucose oxidation was obtained with acetate.

### Discussion:

The tendency towards a reduction in glucose uptake was possibly due to an accumulation of glycolytic intermediaries secondary to impaired glucose oxidation. The tendency to find increased incorporation of glucose label in glycogen indicates that the reduction in oxidation was probably not due to dilution. Palmitate in this concentration (400  $\mu$ M/L.) inhibited glucose oxidation as effectively as the higher concentration previously used.

The decrease of glucose oxidation caused by acetate has its counterpart in intact, non-fasted rabbits (Wick and Drury, 1952). Although acetate is not a normal intermediary, it is activated in a similar fashion to fatty acids (cf. Krebs and Lowenstein, 1960), and it is tempting to compare the similar effects of palmitate and acetate on glucose metabolism. Such a comparison could suggest that palmitate produced its effect at the two-carbon fragment level.

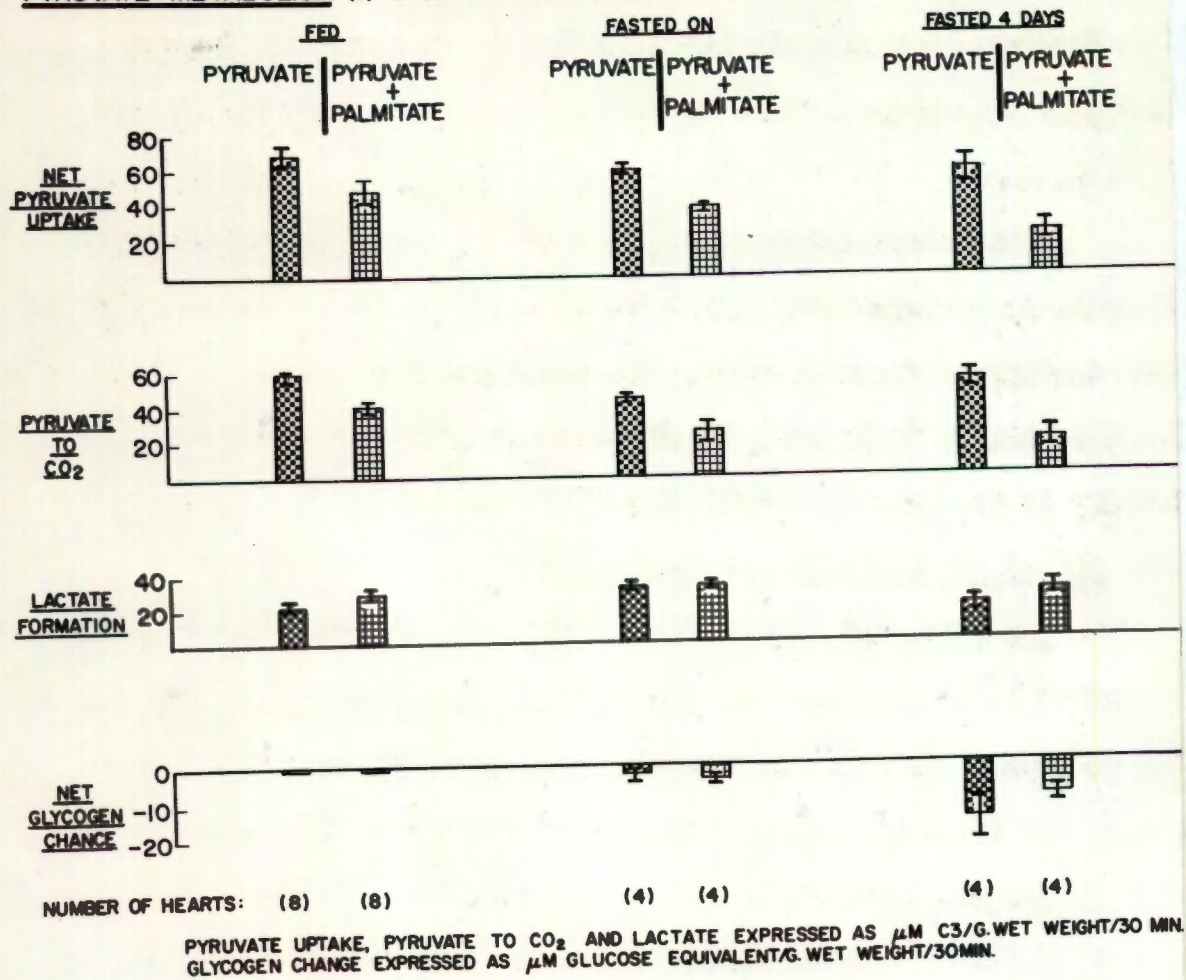
### (II) Effect of Glucose and Acetate on Palmitate-1- $C^{14}$

#### Results:

The addition of glucose 5 mM. to palmitate-1- $C^{14}$  certainly did not depress the percentage of palmitate label incorporated into  $C^{14}CO_2$  (Fig. 34). In another experiment with six hearts in each group, the addition of glucose 10 mM. increased the incorporation of palmitate label into  $C^{14}CO_2$  from  $42 \pm 6\%$  to  $62 \pm 4\%$ , while the incorporation into tissue fatty acid was unchanged ( $20 \pm 2$  vs.  $22 \pm 6\%$ ). In contrast to these observations, the addition of acetate 5 mM. decreased the incorporation of palmitate label into  $C^{14}CO_2$  from  $58 \pm 5\%$  to  $34 \pm 2\%$ . Acetate increased the incorporation into tissue fatty acids from  $21 \pm 2$  to  $33 \pm 3\%$ .



**PYRUVATE METABOLISM (1): Effect of Palmitate  $750\mu\text{Eq/L}$  on Pyruvate- $1\text{-C}^{14}$   $5\text{mM/L}$ .**



**Fig. 35**  $\text{CO}_2$  formation from pyruvate- $1\text{-C}^{14}$ ,  $5\text{ mM/L}$ , depressed by palmitate. Most marked effect in fasted state.



### Discussion:

The effect of glucose on incorporation of label from palmitate into  $C^{14}O_2$  was therefore variable. In the fed state (Fig. 31)  $C^{14}O_2$  formation was reduced by one-third; in hearts from rats fasted overnight,  $C^{14}O_2$  formation was slightly but significantly enhanced by glucose; while, after a four-day fast,  $C^{14}O_2$  formation was unaffected by glucose (Fig. 31).

Acetate reduced glucose oxidation far more than it did that of palmitate, again suggesting that palmitate-glucose interaction was enacted at the two-carbon fragment level. The increased incorporation of palmitate label into tissue fatty acid, in the presence of acetate, was possibly secondary to the impaired palmitate oxidation.

### (III) Effect of Palmitate on Pyruvate-1- $C^{14}$

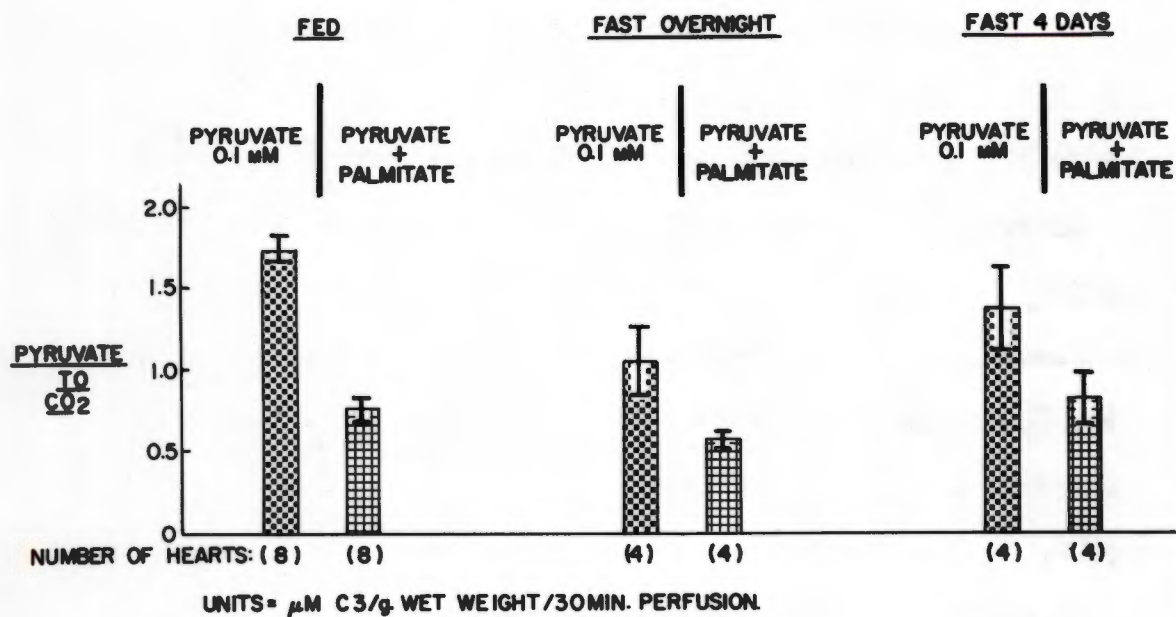
The effect of pyruvate 800  $\mu M/L$ . on pyruvate-1- $C^{14}$  5  $\mu M/L$ . was studied in hearts from rats fed ad lib., fasted overnight, and fasted for four days (Fig. 35). In the fed group, about 85% of the perfusate pyruvate was taken up; in the group fasted four days, 65% was taken up. In all groups palmitate inhibited the formation of  $C^{14}O_2$  from pyruvate-1- $C^{14}$  and reduced the pyruvate uptake. These effects were most obvious in the group fasted for four days. The addition of palmitate tended to increase lactate production. Virtually no counts were recovered in glycogen.

The sum of lactate formation and  $C^{14}O_2$  production exceeded the pyruvate uptake, even allowing for any glycogen breakdown that occurred. The carboxyl-labelled pyruvate exhibited spontaneous decarboxylation during a dummy perfusion, from which it was estimated that at least 7-14% of pyruvate underwent this change. The magnitude of the reduction in pyruvate oxidation caused by palmitate was therefore greater than indicated in Fig. 35.



# PYRUVATE METABOLISM (2)

Effect of Palmitate 750  $\mu$ Eq/L on Pyruvate-1- $C^{14}$   
0.1 mM/L and Glucose 5 mM/L.



**Fig. 36** Palmitate reduced oxidation of pyruvate-1- $C^{14}$  0.1 mM/L.



In an attempt to label intracellular pyruvate, the effect of palmitate on pyruvate 0.1 mM/L. together with glucose 5 mM/L. was studied. As indicated in Fig. 36, palmitate again inhibited the incorporation of the carboxyl label into  $C^{14}O_2$ . Again, no correction for spontaneous decarboxylation was made. The inhibitory effect of palmitate was no greater in the fasted state.

### Discussion:

The avid myocardial uptake of pyruvate confirms the work of Braun-Menendez and co-workers (1939) who used an isolated dog heart. These workers also inferred that cardiac glycogen was not formed from pyruvate.

Studies of pyruvate-1- $C^{14}$  oxidation have the apparent advantage of delineating a single process, namely the intricate reaction involving thiamine (Peters, 1936), coenzyme A, lipole acid and D.P.N. (cf. Krebs and Lowenstein, 1960), by which acetyl CoA is formed from pyruvate. The complex kinetics of such enzymatic decarboxylations has been reviewed by Ochoa (1951).

An inhibition of pyruvate decarboxylation could not be due to dilution of pyruvate because the palmitate does not normally form pyruvate in its breakdown; nor could it be due to an enhancement of the total  $CO_2$  production because the total number of  $CO_2$   $\mu M$ . in the equilibrated system is about 600; to reduce  $C^{14}O_2$  formation from any substrate by half would require the addition of a further 600 unlabelled  $CO_2$   $\mu M$ ., which would correspond to the uptake and complete oxidation of 37.5  $\mu M$ . of palmitate. This compares with the actual F.F.A. uptake of 6 - 7  $\mu M$ . in this experiment. Furthermore, Williamson (1960) showed a constant oxygen uptake in the isolated perfused rat heart irrespective of the substrate used, thereby



implying that the total  $\text{CO}_2$  production is fixed.

On the other hand, if pyruvate were to enter the Krebs cycle by another route than terminal decarboxylation and thereby produced labelled cycle intermediaries, then such labelled cycle intermediaries could be diluted by unlabelled intermediaries from fatty acid oxidation. This possibility is raised by the work of Freedman and Graff (1958), who studied pyruvate metabolism in livers from rats fed with glucose and fasted for 40 hours. They found that concurrent with adaptation to fasting, pyruvate decarboxylation decreased and instead pyruvate chiefly formed dicarboxylic acids by  $\text{CO}_2$  fixation. Since the enzyme for malate formation from pyruvate has been found in heart muscle (Ochoa, 1955) changes similar to those in liver could have occurred in perfusions with both pyruvate and palmitate. Dilution by the unlabelled products of fatty acid degradation then becomes a possibility, but only in the fasted state.

However, if dilution is to be involved it should be a bilateral effect. In other systems such as diaphragm from fed rats, and in liver, pyruvate stimulates rather than inhibits fatty acid oxidation (Fritz and Kaplan, 1960; Weinhouse et al., 1949).

Furthermore, the reduction in pyruvate uptake and the tendency to increased lactate formation, induced by the palmitate, argue against dilution.

Pyruvate, being a glycolytic intermediary, should not accumulate (Krebs and Lowenstein, 1960). It did, however, accumulate in two conditions: (a) after prolonged fasting, when glucose 5 mM was presented to the heart (cf. p. 20); and (b) in the fed state, when glucose and fatty acid were simultaneously present in the perfusate (Fig. 27). These two states could



have had one common factor in operation. In the former condition, the heart was possibly "adapted" to high plasma F.F.A. levels; similar adaptive changes are discussed by Man and Albrink (1957). In the second case, high F.F.A. levels were present in the perfusate. In both cases, F.F.A. inhibition of pyruvate oxidation would seem to provide a plausible mechanism for pyruvate accumulation. "Adaptation" to high plasma F.F.A. levels in fasting could not, however, explain why the oxidation of pyruvate-1- $C^{14}$  (5 and 0.1 mM/L.) was nearly as vigorous in the fasted as in the fed state.

Apart from these speculations, it is evident that an action on pyruvate decarboxylation would explain the increase in lactate formation during the inhibition of glucose oxidation by palmitate.

(iv) Summary

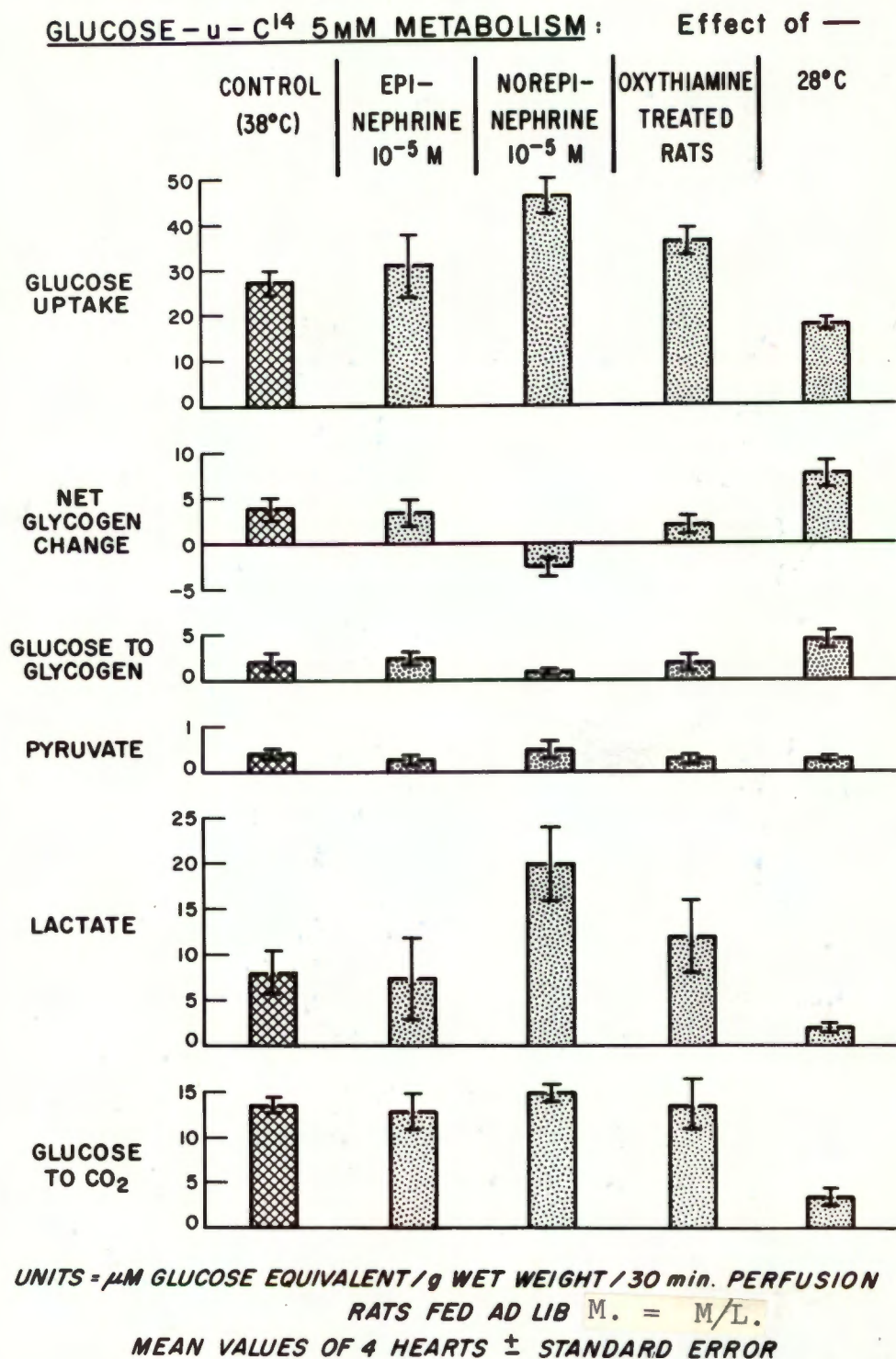
In rats fasted overnight, it was found that:

- (i) Equimolar acetate most closely mimicked the effect of palmitate on glucose-u- $C^{14}$  metabolism;
- (ii) Glucose 5 and 10 mM/L. did not depress palmitate-1- $C^{14}$  oxidation but rather tended to enhance it.

In both fed and fasted states, it was found that:

- (i) Palmitate reduced  $C^{14}O_2$  formation from pyruvate-1- $C^{14}$ .





**Fig. 37** Norepinephrine increased glucose uptake and glycogen breakdown; hypothermia decreased glucose uptake and enhanced glycogen synthesis.



## CHAPTER EIGHT

### THE EFFECT OF ANOXIA, HYPOTHERMIA, EPI- and NOR-EPINEPHRINE ON MYOCARDIAL INTERMEDIARY METABOLISM

#### A. Effect on Carbohydrate Metabolism

The metabolism of glucose- $u\text{-C}^{14}$ , 5 mM/L., was studied in the following conditions: Anoxia (Fig. 13), perfusion at  $28^{\circ}\text{C}$ ., the addition of epi- and nor-epinephrine ( $10^{-5}$  M/L.), and hearts taken from oxythiamine-treated rats. In the latter case the hearts were studied 4 - 6 hours after the rats had received an intraperitoneal injection of 0.5 or 1.0 mg. of oxythiamine, a thiamine antagonist which is reported to cause changes in pyruvate and lactate metabolism closely resembling those found in thiamine-deficient rats (Frohman and Day, 1949). Oxythiamine treatment produced no changes beyond a barely significant increase in glucose uptake. It will receive no further comment, nor will ouabain, which in a concentration of 1  $\mu\text{g}/100\text{ ml}$ . produced no effect.

The following results were of greater interest (Fig. 37):

#### (1) Anoxia

As indicated earlier (p. 17), anoxia increased glucose uptake, net glycogen breakdown and lactate production; it greatly diminished incorporation of label from glucose into glycogen and  $\text{C}^{14}\text{O}_2$ .

The effect of anoxia in stimulating both glucose transport across the cell membrane and glucose phosphorylation has been fully analyzed by Park and Morgan (Park et al., 1959; Morgan et al., 1959).



The rapid glycolysis, increased lactate production and diminished glucose oxidation all indicate that, as expected, anaerobic glycolysis was the major energy source. The data of Williamson (1960) permit the deduction that, in the fed state, anoxia would reduce cardiac glycogen to insignificant amounts within 4-5 minutes of the start of an anoxic perfusion. Thereafter anaerobic glycolysis is dependent on the enhanced glucose uptake. Hearts from fasted rats would take longer before glycogen-depletion, since the rate of glycogen breakdown is not influenced by the initial level of cardiac glycogen (Michel et al., 1959).

Anaerobic metabolism is inefficient. For an instance, the breakdown of one glycogen unit to lactate is accompanied by the formation of 3 units of A.T.P.; on the other hand, oxidative phosphorylation of 2 units of pyruvate via the Krebs cycle yields 30 high-energy phosphorylations (Lehninger, 1954). In the dog heart anoxic glycogen-depletion occurs less rapidly (Danforth et al., 1960). The decrease is largely recovered as hexose monophosphate or lactate, with phosphofructokinase being the rate-limiting enzyme between the two.

As lactate formation occurs so readily in anoxia (Fig. 13), the generous lactate production during a normally oxygenated perfusion of glucose 5 mM/L. might be taken as an indication of a slightly hypoxic system. This was regarded as unlikely because lactate formation occurred in conditions of net glycogen synthesis (Figs. 20 & 21), and because of the physical and metabolic distinctions between normal and under-oxygenation (Fig. 13). Lactate production in aerobic conditions was rather viewed as an indication that the pyruvate-lactate ratio had shifted in favour of lactate due to the initial absence of lactate from the perfusion medium; any lactate



formed was free to diffuse extracellularly. The fallacy of regarding lactate production by muscle of evidence of hypoxia is discussed by Andres and co-workers (1956), who, in a study of forearm muscle metabolism found no correlation between lactate production and oxygen uptake.

To complicate the assessment of lactate formation is the evidence that, in heart muscle, hitherto undescribed pathways exist both for lactate oxidation and possibly for its transformation into glycogen (Miller and Olson, 1954; Edwards et al., 1954 a).

#### (ii) Norepinephrine

Norepinephrine caused the hearts to beat faster (over 300 beats/min.), more forcefully and with a higher coronary flow (about 15 ml./min.). Glucose uptake, net glycogen breakdown and lactate formation increased; incorporation of label from glucose into  $C^{14}O_2$  was unchanged, while less label was incorporated into glycogen. Epinephrine caused all these effects in only one of four hearts.

The increased cardiac force and rate are similar to the effect of norepinephrine on the isolated heart of the rabbit, guinea-pig and dog (Leusen and Essex, 1953). In the isolated perfused rabbit heart the effect of norepinephrine on coronary flow is variable. Usually there is an increase (Lu and Melville, 1951). Owing to important species-differences, these effects on cardiac performance should not be directly translated to man (Lands and Howard, 1952).



Cruickshank (1913) found that epinephrine affected the dog heart-lung preparation by increasing the disappearance of sugar from the blood; glycogen breakdown was due to "exaggerated work of the heart". Glycogenolysis was also found by Bogue and co-workers (1939). More recently Hess and Haugaard (1958) used an epinephrine concentration of  $5 \times 10^{-6}M$  to demonstrate that epinephrine directly influenced cardiac phosphorylase. There was a shift in the equilibrium of phosphorylase from type b (inactive) to type a (active). Presumably norepinephrine has a similar but more marked effect.

The effect of norepinephrine on glucose uptake is definite but not easy to explain. There may be an analogy to the increased glucose uptake induced by increased cardiac work in the isolated rat heart (Bleehen and Fisher, 1954).

The finding that epi- and nor-epinephrine increase cardiac glycogen in the intact rat (Evans, 1933; Bloom and Russell, 1955) can readily be explained by the ability of these agents to mobilize F.F.A. from adipose tissue. Increased cardiac uptake of F.F.A. could then result in diversion of glucose from oxidation to glycogen synthesis (cf. Fig. 28).

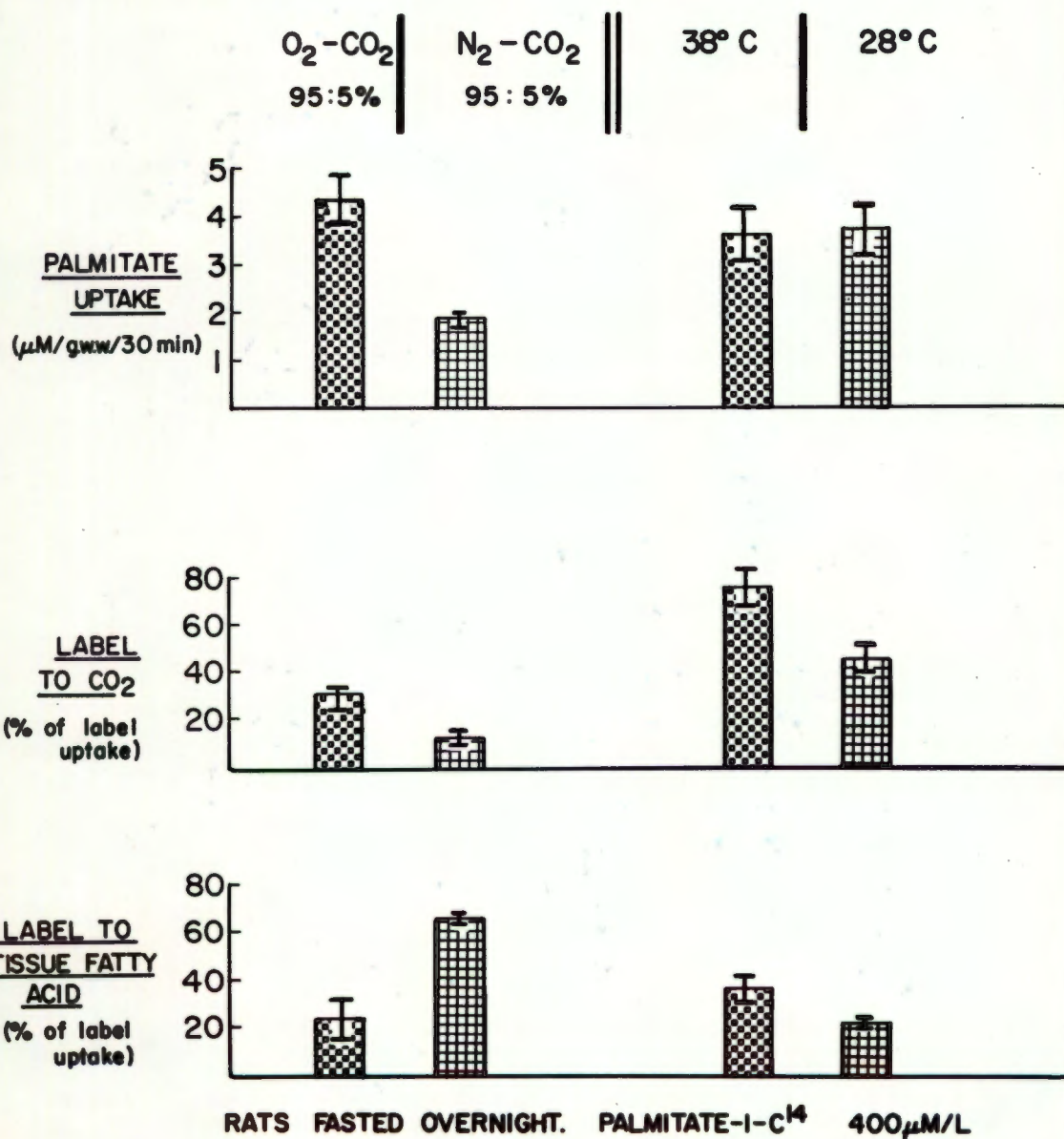
### (III) Perfusion at 28°C.

The heart rate fell to about 150 beats/min. but force and coronary flow were unaltered. Glucose uptake was reduced, but this could not account for the 70 - 75% reduction in lactate formation and incorporation of label from glucose into  $^{14}CO_2$ . The incorporation of label into glycogen and net glycogen synthesis were increased.

The effect of hypothermia in reducing glucose uptake by heart muscle was reported by Edwards and co-workers (1954 b). In the diaphragm,



# **PALMITATE METABOLISM (6): Effect of Anoxia and 28°C.**



**Fig. 38** Anoxia reduced palmitate uptake; hypothermia did not affect uptake. Both reduced CO<sub>2</sub>-formation.



Kipnis and his co-workers (1959) found that cooling reduced glucose utilization more than glucose uptake. The marked change in glucose fate from  $\text{CO}_2$  and lactate formation to glycogen synthesis has apparently not been reported before.

Superior electrolyte stability of the isolated perfused rat heart at low temperatures is reported by Taylor (1961), who routinely employs  $27.5^\circ\text{C}$ .

### B. Effect on Palmitate Metabolism

Only the effect of anoxia and of perfusion at  $28^\circ\text{C}$ . were studied.

#### (i) Anoxia

Anoxia produced the usual deterioration in physical function of the heart. Furthermore, it diminished the proportion of label oxidized and increased the amount recovered as tissue fatty acid (Fig. 38). Over 80% of the  $\text{C}^{14}\text{O}_2$  formation took place in the first half of the perfusion period, probably because the pre-perfusion had been with oxygenated buffer. D.P.N., essential for fatty acid oxidation, is rapidly reduced in anoxic heart muscle (Michal et al., 1959). This stresses the inability of the anoxic myocardium to oxidise fatty acids and emphasizes the importance of anaerobic glycolysis.

#### (ii) Perfusion at $28^\circ\text{C}$ .

The palmitate uptake was unaltered, but incorporation of label into  $\text{CO}_2$  and tissue fatty acid was diminished. The decreased recovery of label needs further confirmation.

### C. Summary

It was found that:

- (i) Anoxia increased glucose but decreased palmitate uptake;



- (II) Norepinephrine caused an increase in cardiac rate, force and coronary flow; it also increased glucose uptake, lactate production and glycogen breakdown;
- (III) Hypothermia ( $28^{\circ}$ ) decreased glucose but not palmitate uptake, and altered the fate of the label in both instances.



## CHAPTER NINE

### REVIEW OF DATA

The aim of the work reported here was to delineate pathways of myocardial intermediary metabolism, using the isolated perfused heart as a unique experimental preparation. These pathways will now be reviewed.

#### A. SUBSTRATE UPTAKE

The isolated perfused rat heart has a wide range of substrates, corresponding to the "great versatility" of the human heart which Bing (1955) calls the biochemical "safety factor" in myocardial metabolism.

Glucose uptake by the perfused rat heart was increased by a higher perfusate glucose concentration (Fig. 17), the fed state (Fig. 18), anoxia (Fig. 13), insulin (Table 10) and norepinephrine (Fig. 37). Uptake was decreased by lower perfusate glucose concentrations (without any "glucose threshold"), and by the fasted state, hypothermia (Fig. 37), and the simultaneous presence of pyruvate in the perfusate (Fig. 33). Palmitate (Fig. 29) or aceto-acetate (Williamson, 1960) when present together with insulin, suppressed the effect of insulin on glucose uptake.

Many of the factors controlling uptake of glucose have been exhaustively studied by Bleehen and Fisher (1954) and Morgan and co-workers (1961). The most important contribution of the present data is to stress the importance of exact control of the donor rat's nutritional state in studies of cardiac glucose metabolism.

Mannose uptake was of the same order as glucose uptake and insulin-sensitive.

Fructose uptake was about one-fifth that of glucose; its



insulin-sensitivity was abolished by equimolar glucose. The small fructose uptake argues against suggestions that this hexose could be an important fuel for muscle metabolism.

Hearts from fed rats extracted nearly all the pyruvate present in the perfusate (Fig. 35). There was less vigorous uptake in the fasted state. In either nutritional state palmitate diminished the uptake.

Acetate inhibited both glucose and palmitate oxidation (Figs. 33 and 34). It was therefore taken up from the perfusate, and mechanisms for its activation must be present in the myocardium.

Palmitate uptake was linearly related to the perfusate palmitate concentration at levels corresponding to those found in fed and fasted rats (Figs. 14 and 25). Uptake was influenced by the physico-chemical properties of the albumin to which the palmitate was bound. Anoxia and the simultaneous presence of acetate in the perfusate both decreased palmitate uptake. Neither glucose nor hypothermia (28° C) affected the uptake.

Oleate uptake slightly exceeded that of palmitate.

Although F.F.A. uptake in vivo has been well demonstrated (Ballard et al., 1960), the present studies are apparently the first to delineate clearly the effect of the concentration of F.F.A. on its uptake and to stress the vigorous myocardial uptake of F.F.A. in the fed state.

The rat heart is also known to take up the following substrates not studied in these experiments: lactate, aceto-acetate and B-hydroxybutyrate (Williamson, 1960), and tripalmitin from palmitate-labelled chylomicrons (Bragdon and Gordon, 1958).



## B. INTRACELLULAR FATE OF GLUCOSE

### (1) C<sup>14</sup>O<sub>2</sub> Formation

By the use of the low volume closed re-circulation system, it has been possible to relate substrate uptake to C<sup>14</sup>O<sub>2</sub> formation and to assess some factors controlling C<sup>14</sup>O<sub>2</sub> formation. These studies would appear to be the first to define such inter-relations in an intact, contracting heart muscle preparation.

The formation of C<sup>14</sup>O<sub>2</sub> from randomly-labelled glucose was governed by the following factors:

#### (a) The Nutritional State of the Donor Rats.

For the same glucose uptake, the incorporation of glucose label into C<sup>14</sup>O<sub>2</sub> was up to three times as much in hearts from rats fed ad lib., as in hearts from rats fasted overnight (Fig. 19). The cause of this important difference is not obvious, but some factors to consider are: "adaptation" to non-carbohydrate metabolism in the fasted state; increased glycogenolysis in perfused hearts from fasted rats; and any direct effect of fasting on glucose uptake.

#### (b) The Glucose Uptake.

The proportion of glucose oxidized was about 50% at lowest uptakes and under 20% at highest uptakes (Fig. 19). Studies with varying perfusate concentrations of glucose showed that even at physiological concentrations (5-10 mM/L) a plateau was reached in C<sup>14</sup>O<sub>2</sub> formation from glucose, suggesting that the rate of glucose uptake outstripped the oxidative capacity of heart muscle. The glucose which was not oxidized chiefly formed lactate and



glycogen, but at highest uptakes the decreased carbohydrate recovery (Table 9) suggested some other fate for the glucose. Park and his associates (1959) found an accumulation of free intracellular glucose with very high glucose uptakes.

(c) Oxidative Capacity of the System.

In both the fed and the fasted states a plateau was reached in  $C^{14}O_2$  formation from glucose label. The factors regulating oxidative phosphorylation in muscle mitochondria have recently been reviewed (Siekevitz, 1959), and muscle appears to share with many other kinds of respiring cells (cf. Krebs and Lowenstein, 1960) the obligatory coupling of electron transport to the phosphorylation of A.D.P. The availability of A.D.P. could therefore be one rate-limiting step in heart mitochondrial oxidation. Myocardial regeneration of A.D.P. is intimately connected with the reaction between actin and myosin (Fig. 11). Also controlling the rate of oxidation in the Krebs cycle are factors concerned with the rate of oxalacetate formation, the supply of acetyl CoA, and possibly the availability of the condensing enzyme; these three factors are thought to control the rate of citrate formation which is a rate-limiting step in the Krebs cycle (Krebs and Lowenstein, 1960).

The adequacy of the myocardial oxygen supply directly influenced  $C^{14}O_2$  formation from glucose label (Fig. 13). Impaired oxidative phosphorylation at a cellular level was therefore one result of anoxia; associated with this was the gross deterioration in cardiac performance. This stresses a link between intracellular events and behaviour of the whole heart.



## (d) Temperature.

A reduction of  $10^{\circ}$  C. in the perfusion temperature reduced  $C^{14}O_2$  formation from glucose label by 70-75%, a greater effect than could be accounted for by the reduction in glucose uptake. The decreased lactate and increased glycogen formation at this temperature show that the reduction in  $C^{14}O_2$  formation was not due to hypoxia. The low temperature therefore shifted the intracellular fate of glucose in favour of storage of energy as glycogen.

## (e) Insulin.

The increase of  $C^{14}O_2$  formation in the presence of insulin paralleled the increased glucose uptake (Table 10), making it unnecessary to postulate any direct effect of insulin on glucose oxidation.

No attempt was made to define the activity of the hexose monophosphate shunt in this tissue. There is some indirect evidence (p. 38), based on the negligible fatty acid formation from glucose, that the shunt is not well developed in heart muscle.

(ii) Other fates of glucose.

There was significant incorporation of label from glucose into glycogen but not into tissue fatty acids (Figs. 20 and 30). This suggests that glucose is stored as glycogen and not as fatty acid, although it could contribute glycerol to triglycerides. Glycogen formation from glucose was enhanced by the fed state (Fig. 20), by insulin (Table 10), by palmitate, acetate and pyruvate (Fig. 33), and by hypothermia. Glycogen breakdown during the perfusion was enhanced in the fasted state (Fig. 15),



by anoxia (Fig. 13), and by norepinephrine (Fig. 37). No glycogen formation occurred from pyruvate or palmitate.

Lactate formation was minimal at lowest glucose perfusate concentrations and increased with increasing glucose concentrations. Increased lactate formation was associated with increased glycogenolysis during anoxia (Fig. 13), norepinephrine stimulation (Fig. 37), and during the perfusion of hearts from fasted rats (Fig. 15). In hypothermia (Fig. 37) lactate production was depressed in spite of increased glycogen synthesis.

There is a suggestion that at highest glucose uptakes glycogen and lactate formation reached a plateau similar to that found for  $C^{14}O_2$ . In that case, the best explanation would be that phosphorylation had become rate-limiting at such high glucose uptakes.

Lipid formation from glucose is quantitatively minimal, and the abundant endogenous cardiac lipid (Bloor, 1926) must therefore be formed from sources other than glucose.

### C. INTRACELLULAR FATE OF F.F.A.

By the use of radio-active palmitate, the fate of F.F.A. in the myocardial cell can be assessed with much greater accuracy than by methods employing differences in plasma fatty acid levels across the myocardium, without the help of isotopes to trace the fate of the fatty acids.

Using palmitate-1- $C^{14}$ , the major part of the label (up to 75%) was recovered as  $C^{14}O_2$ , and most of the rest as tissue fatty acid. This held for palmitate concentrations up to 1,450  $\mu M/L.$ , but there was evidence that the label was not fully equilibrated with all the unlabelled palmitate, and it is not possible to say whether higher palmitate uptakes saturated the cardiac oxidative mechanisms or not.



The similar metabolism of palmitate labelled with  $C^{14}$  in positions 1, 6, and 11 is important evidence in the validation of studies with carboxyl-labelled palmitate.

Of equal importance is the demonstration that variations in the donor rat's nutritional state did not effect the intracellular fate of palmitate. This is in marked contrast to the findings with glucose.

The exact function of the tissue fatty acid fraction was not defined; the incorporation of palmitate label into this fraction was increased by the simultaneous presence of glucose and acetate in the perfusate. Further studies on the characterization of this fraction and the role of the rich endogenous cardiac lipid stores (Bloor, 1926; Kochen et al., 1960) are needed. That such lipids can be metabolized is strongly suggested by Williamson (1960) who found that, during a substrate-free perfusion of the rat heart, most of the energy was derived from endogenous non-carbohydrate sources. Studies on isolated diaphragm suggest that oxidation of endogenous long-chain fatty acids does occur (Neptune et al., 1960).

#### D. GLUCOSE - F.F.A. INTERACTION

The control of the intracellular fate of glucose- $u-C^{14}$  by palmitate would appear to be a hitherto unexplored concept. That this effect was real, was established by the diversion of glucose from oxidation to other fates, and by the noticeable lack of any consistent effects of glucose on palmitate oxidation. The conclusion is that there was preferential oxidation of palmitate in the conditions of this study.

Similar effects on the metabolism of glucose- $u-C^{14}$  were obtained by equimolar acetate. This led to the study of the effect of palmitate on



$C^{14}O_2$  formation from pyruvate-1- $C^{14}$ ;  $C^{14}O_2$  formation was reduced in all nutritional states, but isotope dilution could not be excluded as a cause of this effect.

Further studies should elucidate this problem. For example, a study of labelled Krebs cycle intermediaries and amino-acids that could be formed from pyruvate, should show whether  $C^{14}O_2$  formation from pyruvate-1- $C^{14}$  is solely through decarboxylation and acetyl CoA formation or whether other routes of entry into the Krebs cycle are available. The measurement of total  $CO_2$  production (labelled and unlabelled) would evaluate the extent of dilution as a cause of the palmitate action on pyruvate-1- $C^{14}$ . This could perhaps best be carried out on tissue slices respiring in a Warburg apparatus.

If F.F.A. blocked glucose breakdown at the site of entry of pyruvate into the Krebs cycle, this would explain the diminished  $C^{14}O_2$  formation from pyruvate-1- $C^{14}$  and also the increased lactate formation during the inhibition of glucose oxidation by F.F.A. (Fig. 2B).

Another site to consider for the action of palmitate from glucose metabolism is at the level of synthesis of triglyceride.  $\alpha$ -Glycerophosphate generation is thought to be related to triglyceride synthesis by acting as an acceptor for the acyl portion of acyl CoA derivatives; the acyl CoA units are thereby redirected from oxidation to triglyceride formation. This chain of events is thought to be of much importance in glucose F.F.A. interaction in adipose tissue (Fritz, 1961).

In heart muscle, glyceride-glycerol formation from glucose-u- $C^{14}$  was greatest when palmitate was also present. In these conditions glucose and not palmitate oxidation was reduced. Therefore glyceride-glycerol formation does not exert control over palmitate oxidation in heart muscle.



Furthermore, the amount of glucose label incorporated into glyceride-glycerol was small compared with its oxidation to  $C^{14}O_2$ , and hence redirection of glucose from oxidation to glyceride synthesis cannot explain the inhibitory action of palmitate.

The intracellular control of glucose metabolism by F.F.A. is secondary to F.F.A. uptake by the cell, a process influenced by the perfusate F.F.A. concentration and albumin binding sites. All F.F.A. studies presented here really represent the effect of an albumin-F.F.A. complex. To ensure that the effects on glucose oxidation are due to the F.F.A. alone could be done by changing the F.F.A. carrier. To elucidate the effect of albumin binding on F.F.A. uptake, the rat heart could be perfused with albumin near a physiological concentration (e.g. : 4g./100 ml.) and the uptake, if any, of the endogenous tightly bound F.F.A. assessed. Attempts to label this endogenous F.F.A. should also show whether exogenous and endogenous F.F.A. can equilibrate or not.

Separation of the two processes of F.F.A. uptake and intracellular usage would be aided by the use of a non-metabolized fatty acid analogue such as 3,3-dimethyl phenylmyristic acid (Goodman and Steinberg, 1958), which does not undergo  $\beta$ -oxidation because of the absence of hydrogen atoms on the  $\alpha$ -carbon.

Such further data would extend the knowledge of glucose-F.F.A. interaction in myocardial metabolism. To translate these conclusions into terms of the intact organism would require definition of the hormonal-metabolic factors influencing F.F.A. and glucose. Of prime importance in the release of F.F.A. by adipose tissue, is the availability of glucose (cf. Cahill et al., 1960; Fritz, 1961).



Glucose utilization by adipose tissue is associated with increased glyceride-glycerol formation and triglyceride synthesis, thereby diminishing release of F.F.A. from adipose tissue into the blood. As indicated in Fig. 14, the fed state in the rat was associated with the high glucose and low F.F.A. levels in blood, with the converse in the fasted state.

In fed humans and dogs, Olson (Fig. 32) found carbohydrate the main energy source, but in the post-absorptive state fatty acids are the chief cardiac fuel (Bing et al., 1958). This continuous oscillation between carbohydrate and fatty acid as source of cardiac energy is clearly defined by Olson and Piatnek (1959). Considering the whole 24 hour period, Bing and his associates (1958) conclude that "the results obtained in the beating human heart in situ show that fatty acids contribute more to myocardial energy metabolism than does any other substrate".

The suggestion presented in this thesis is that the contribution of fatty acids is not only by provision of energy through oxidation, but also by actual intracellular control of glucose fate.



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